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CONTENTS OF THIS ISSUE

Iodine in the Atomic Age 479
George M. Curtis

The Concentration and Purification of Diphtheria Toxoid by Polyphosphate Precipitation 488
Morris B. Jacobs and *Roberta Jacobs Gillis*

Urinary Excretion of the Metabolic Products of Parathion Following Its Intravenous Injection 491
Joseph F. Gardocki and *Lloyd W. Hazleton*

The Relative Stability of Rutin and Quercetin in Alkaline Solution 495
Earl B. Dechene

Assay of Cardiotonic Drugs by a Chick Embryo Method 497
Gordon H. Bryan and *Curtis H. Waldon*

Microtoxicology. IX. The Effect of *para* Substitution in Three New Thiazole Antihistaminics upon Their Reactivity to Microchemical Tests 501
Thomas J. Haley and *Geo. L. Keenan*

Acute and Short-Term Toxicity Studies of Fused Bentonite Sulfur Preparations 503
Harold C. Hodge, Elliott A. Maynard, David R. Hawkins, Edwin H. Church, Harvey J. Blanchet, Jr., Joan F. Thomas, Helen McRae, and *Raymond Kesel*

A New Hydrocarbon Ointment Base: Jelene 50W 509
E. R. Jones and *B. Lewicki*

The Effect of Iodides on Phenobarbital Narcosis 511
John C. Krantz, Jr., and *Mary Jane Fassel*

Determination of Nicotinic Acid in Pharmaceutical Products 513
Alexander Mueller and *Sereck H. Fox*

Studies on the Stabilization of Human Serum Albumin. The Effect of the *p*H, the Stabilizers, and the Albumin 517
John H. Hink, Jr., and *Frederick F. Johnson*

Cellulose Acetate Phthalate as an Enteric Coating Material 520
C. J. Malm, J. Emerson, and *G. D. Hiatt*

Pressor Drugs. II. Rate of Loss in Pressor Potency of Solutions of Epinephrine and Its Analogs During Storage 526
James C. Munch, Aaron B. Sloane, and *Albert R. Latven*

United States Pharmacopœial Convention, Annual Financial Statement 530

The Synthesis of Acetyl-1-C^{14}-Salicylic Acid 532
Jean W. Clappison, J. P. Hummel, W. D. Paul, and *J. I. Routh*

Book Reviews 533

Advertising ..... Cover pages 2, 3, 4, and pp. I–VIII

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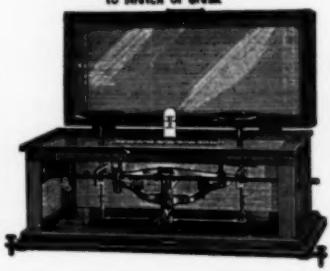

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VOLUME XL — *OCTOBER, 1951* — NUMBER 10 — CONSECUTIVE NO. 20

Iodine in the Atomic Age*,†

By GEORGE M. CURTIS

THAT IODINE is of major consequence in any consideration of the essential nature of thyroid activity, or of thyroid disease, long has been a matter of common knowledge. The thyroid gland is a principal storehouse for iodine. The thyroid hormone has a high iodine content. The use of iodine in the prevention, as well as in the treatment of goiter has become even household conversation. In fact, iodine is now generally regarded as a nutritional necessity. Nevertheless, trite as now may sound this older account, newer facts are constantly being added, with the resultant opening of even wider horizons in our understanding of the evolving story of the clinical significance of iodine.

It is established that iodine is constantly present in human blood. A part actually forms a fraction of the circulating thyroid hormone. The intake of food iodine fluctuates. There ensues a consequent variable loss of iodine from the body by excretion. The level of the blood iodine varies. Moreover, by measuring this variation we may obtain some idea of the secretory activity of the thyroid gland. Indeed, that portion of the blood iodine contained within the circulating thyroglobulin may actually be fractionated away and analyzed separately. This determination has become of considerable clinical value in recognizing variation of thyroid function.

After the development of the cyclotron by Lawrence it became possible (1940) to prepare radioactive iodine for experimental purposes. This was created by bombarding elemental iodine or even an entirely different element, tellurium, with high-speed protons. The biologic use of radioiodine has already added greatly to our knowledge of the fundamental physiology of iodine as well as its utilization by the thyroid gland in health and disease. The use of one of the radioactive isotopes of iodine, I^{131}, as a "*tracer*" or in the preparation of "*autographs*," has been most fruitful in adding to our knowledge of endogenous iodine metabolism.

It is the purpose of this address to present a brief sketch of the background history of biologic iodine, thus better to orient ourselves to the more recent developments. Then, to outline those principles underlying the human iodine requirement; to present certain features of the metabolism of iodine as related to nutrition as well as to thyroid function in health and disease; to demonstrate something of the action of radioiodine; and finally to consider certain of the newly discovered principles which underlie the clinical use of radioiodine in the treatment of thyroid disease.

* Received July 16, 1951, from the Department of Research Surgery, Ohio State University, Columbus, Ohio.

† Presented to the Scientific Section of the A. PH. A., Atlantic City meeting, May, 1950, by Dr. George M. Curtis, recipient of the second (1949) Chilean Iodine Educational Bureau Award for outstanding research in the chemistry and pharmacology of iodine.

HISTORIC BACKGROUND

The ancients used burnt sponge and seaweed in the treatment of goiter. This was a fortunate empiricism as the nature of goiter was but little understood and iodine was unknown. Iodine was discovered in 1811 by a French pharmacist, Courtois (1). Napoleon was blockaded in France by the British fleet. It was consequently impossible for him to import sufficient foreign nitrates to make gunpowder. He appealed to his scientific staff who made it possible to process the cruder local supplies of available nitrate by treatment with a lye made from the ash of seaweed. This was carried out in large copper vats. Some unknown substance corroded the vats. Upon investigation this proved to be a new element, iodine. Thus, like the development of the bomb, the discovery of iodine resulted from the stress of military necessity.

Soon afterward Sir Humphrey Davy demonstrated the presence of iodine in sponges, seaweed, and various other forms of marine life. As a logical sequence, a British physician William Prout (2) prescribed the new medication, iodine, as early as 1816 in the treatment of goiter. Two Swiss physicians, Coindet de Génève (3) and Straub von Bern used iodine as early as 1820 for a similar purpose. Since that time *iodine and the goiter problem have become, and remain, inseparable.*

In 1831 a young French chemist, Boussingault, recorded that in the Andes, where goiter is prevalent, the sea salt of Guaca was used as a "cure." Moreover, that the districts in which an iodine-rich salt was used, were free from goiter. This was followed by other similar investigations, resulting in the development of Prevost's theory, and its announcement in 1849. Prevost maintained that there was a direct relation between the supply of iodine and the incidence of goiter. His theory was further substantiated by the subsequent extensive chemical studies of Chatin (4). Chatin published a series of pertinent papers in the "*Comptès Rendùs*" between 1850 and 1876. These presented the best supporting evidence available up to that time.

As a consequence of Chatin's findings, three French provinces attempted iodine as a preventive against the development of goiter as early as 1860. The results were not satisfactory. It is not so difficult to understand the ill effects of that extensive experiment in the light of recent investigation. Anyway, the use of iodine as a goiter prophylactic was even denounced before the French Academy of Medicine by Rilliet, and rapidly fell into disrepute. The general use of iodine as a preventive against the development of goiter thus received a severe setback. During the ensuing thirty-five years other theories arose as to the nature of goiter. It was during this thirty-five year period (1860–1895) that the infection theory of the origin of goiter gained credence. This was doubtless due to the wide attention attracted by the contemporary discoveries of Louis Pasteur.

Theodore Kocher (1841–1917), pioneer student of the surgical problems concerning goiter, was well aware of the alleged relation between iodine deficiency and subsequent enlargement of the thyroid gland. In fact, he assigned to one of his assistants the problem of investigating the chemistry of the thyroid gland, to see whether or not it contained unusual amounts of iodine. The assistant, using the inadequate methods of biologic iodine analysis available at that time, failed to find iodine and thus missed making a discovery of fundamental significance. Sometime later, Kraske, for whom the Kraske operation was named, at that time professor of surgery at the University of Freiburg, asked one of his colleagues to make a similar investigation. Emil Baumann (5) succeeded, and demonstrated in 1895 that there were *quantities* of iodine within the human thyroid gland. As final proof Baumann actually isolated and demonstrated iodine before one of his scientific societies, in its characteristic violet vapor form, in tubes. The significance of such a major contribution was widely appreciated.

There consequently ensued a widespread renewal of interest in the interdependence of iodine and the thyroid gland. Again iodine was used preventively in the treatment of goiter. David Marine (6) and his associates obtained significant results in 1917 in Akron, Ohio, by administering small amounts of iodide to schoolgirls. Other investigators in other countries were likewise successful.

Several earlier studies had resulted in the surmise that the thyroid gland actually takes up iodine and fashions it into a hormone, and that *probably* the thyroid hormone actually contained iodine. These researches were carried to their logical consummation by Kendall (7), who actually used *quantities* of beef thyroids in isolating in 1914 a small amount of crystalline substance. After subsequent extensive chemical investigation he called this substance thyroxine and designated it as the secretion of the thyroid gland. Kendall's thyroxine contained 65% iodine. With his associates he attempted the formula of thyroxine but was unsuccessful. As a consequence he could only isolate it by extraction from the thyroid gland and was not able to synthesize it.

Harington and Barger working in London noted certain discrepancies in the given chemical composition (8). As a consequence they first isolated sufficient quantities of pure thyroxine, using methods giving a considerably greater yield, and then determined its true chemical structure. Working from this formula they were actually able to synthesize the hormone of the thyroid gland outside of the animal body.

NEED OF AN ADEQUATE MICROMETHOD

It thus became clear that, in order to study further the metabolism of iodine in the human body, biochemical micromethods must be developed, so accurate that they would determine quantitatively the minute amounts of iodine ordinarily present biologically. True, the amount of iodine in the thyroid gland was high. On the other hand, the amount normally present within the ingesta, within the blood, the living tissues and excreta was truly minute. Chatin had learned this in his earlier extensive studies.

The thyroid iodine was readily determined by employment of the older, coarser methods, owing to its relatively high concentration (31). This did not hold, however, for the blood and tissues. Davy had originally separated and determined the iodine present in sponges, by ashing the organic material.

The principle of his method has been widely followed. For many years methods were devised for the determination of the iodine content of ordinary foods. None of these, however, was sufficiently delicate *even to detect* the minute amount of iodine present within the quantities of human blood ordinarily used for chemical analysis.

Thus, advancing investigation into the details of human iodine metabolism created an acute demand for an adequate biologic micromethod. In 1922 Theodore von Fellenberg, chemist to the Swiss government, developed such a procedure (9). This would determine one ten-thousandth of one milligram[1] (0.0001 mg.) or one three hundred-millionth of an ounce of iodine. Facilities were thus available to commence an extensive investigation of iodine as it occurs in nature, and particularly in its relation to man.

HUMAN REQUIREMENT OF IODINE (17)

The quantity of iodine necessary to answer the daily needs of the human body should be sufficient to meet the daily losses by excretion, as well as maintain within the body such reserves as may be required in the manufacture and distribution of an adequate supply of thyroid hormone. The amount of iodine intake, however, and particularly in certain geographic regions, is not always equal to the physiologic needs. This is in contrast with the organism's chlorine requirement, which is more readily met because of the association of this element with the sensation of taste.

The fundamental question thus arises: How much supplemental iodine is necessary in those regions in which goiter is prevalent, in order to protect the populace from the effects of iodine deficiency? Thus far, three methods have been developed in attempts to answer this question.

By the *geographic method* the iodine intake of the inhabitants of goiter-free areas is determined and compared with that of areas of varying degrees of goiter incidence. The difference in the amount of average iodine intake is then regarded as the amount of supplemental iodine required. According to von Fellenberg's reckoning the annual iodine intake in one goitrous and one practically goiter-free area in Switzerland was 4.7 and 11.4 mg., respectively (9). Calculating from this, the iodine requirement would be in the neighborhood of 30 μg. daily, a figure now regarded as extraordinarily low. Estimating on the basis of determinations of the average daily urinary loss of iodine, which is an unusually accurate barometer of the iodine intake of a given area, the daily iodine requirement would lie somewhere between 100 and 200 μg. (10, 15) (Table I).

The principle of *thyroxine formation and decay* was originally outlined by Plummer and Boothby (11) and subsequently developed by Thompson (12). Plummer and Boothby observed that the daily rate of thyroxine decay ranged between 200 and 400 μg. Thus, a daily supply of thyroxine in this quantity maintained a normal basal metabolic rate in a "totally myxedematous patient." Thompson and his group concluded that from 300 to 400 μg. of thyroxine was necessary to maintain a normal basal metabolic rate in myxedematous patients at bed rest. On the basis of these results the amount of thyroxine supplied daily to the circulation by the thyroid gland, in order to maintain normal metabolic activity, is equivalent to from 130 to 260 μg. of iodine. The uncertain factor here, however, is that iodine-containing end products of thyroxine decay may be retained and eventually re-utilized by the thyroid gland in the further synthesis of thyroid hormone.

Studies of the *total iodine balance* (Figs. 1, 2, 3 and Tables II and III) constitute the third principle which has been employed. The iodine balance represents the daily amount of iodine lost or retained by the body, as ascertained by the difference between the amount of iodine intake and excretion (Fig. 1). Pioneer determinations of iodine balance were accomplished by von Fellenberg (9), who reported low values and consequently a *low* daily requirement. The balance studies of Scheffer (13)

[1] 0.001 mg. is designated a microgram, or often a *gamma*.

TABLE I[a].—THE NORMAL DAILY HUMAN URINARY IODINE EXCRETION IN VARIOUS GEOGRAPHIC REGIONS (10)

Geographic Region	Investigator	Range in Daily Urinary Iodine Excretion, μg.	Av. Daily Urinary Iodine Excretion, μg.
Nongoitrogenic Regions			
Danzig	Liek	200–590	343
Berlin, Germany	Scheringer (17 women)	66–389	141
	(7 men)	103–214	173
Vik-i-Sogne, Norway	v. Fellenberg	94–240	146
New Orleans, La.	Moore[b]	60–270	117
Forte dei Marmi, Italy	v. Fellenberg	30–140	72
Av. normal 24-hr. urinary iodine (in five "goiter-free" regions)			165
Goitrogenic Regions			
Effingen, Switzerland	v. Fellenberg	28–108	64
Columbus, Ohio	The authors	7–196	51
Sandsvaer, Norway	Lunde	6– 83	38
Lwow, Poland	Elmer and Scheps	34– 40	36
Pecs, Hungary	Scheffer	21– 33	27
Av. normal 24-hr. urinary iodine (in five goitrogenic regions)			42

[a] Note the great variability in the daily urinary loss; however, the diminished average excretion in the goitrogenic regions.

[b] We are indebted to Margaret C. Moore, of New Orleans, La. for permission to include these data.

TABLE II[a]—THE HUMAN IODINE BALANCE DURING PREGNANCY

Period	Date Started	Weight, Kg.	Iodine — Output — Urine, μg.	Feces, μg.	Sweat, μg.	Total, μg.	Intake, μg.	Balance, μg.	Date	Blood Iodine, μg. %	BMR, %	Remarks
I	2-13-37	64.5	163	28	25	216	104	−110	2-12-37	7.4	+7	General hospital management
									2-15-37	5.9	+8	
II	2-16-37	64.1	194	18	31	243	105	−138	2-17-37	10.9	+5	
III	2-19-37	64.5	166	32	18	216	77	−139	2-22-37	5.6	+3	

[a] Woman of 26, pregnant eighth month; dietary intake 2,170 calories with 49 Gm. of protein (14). Note high negative balance on a low iodine intake.

made in Pecs, Hungary, revealed that 54 μg. of daily iodine intake was sufficient to maintain the iodine balance in a normal person.

Ohio State University studies (Figs. 1, 2, 3 and Tables II and III) were made on normal persons maintained at bed rest on a monotonous diet low in iodine content, under controlled hospital conditions (14). Under these circumstances the *basal* human adult iodine requirement was found to range from 44 to 75 μg. daily and to average 67 μg., or approximately 1 μg./Kg. of body weight. This average daily requirement is comparable to that determined by Scheffer (13). However, it should be emphasized that it applies to adults maintained under controlled *basal* conditions. Moreover, to arrive at an *optimal* iodine requirement, it is necessary to take into account individual activity as well as the varied stress and strain of existence (16).

After consideration of the difference in iodine intake between goitrogenous and nongoitrogenous regions (Table I) and the amount of iodine estimated as necessary to maintain normal metabolic activity, 2 μg. daily per Kg. of body weight, together with the daily basal requirement of 1 μg., can be reasonably justified as an amount sufficient to account for basal needs, those of ordinary activities, and also some for reserve. The *optimal* daily requirement would thus be somewhere near 200 μg. for an adult of 70 Kg., a value compatible with Elmer's deduction from various investigations that the human *optimal* requirement ranges between 100 and 200 μg. daily (16). The pregnant woman should receive additional iodine (Table II). A sufficient amount will be supplied by the daily use of iodized salt (17).

Various methods of supplying supplemental iodine to the inhabitants of iodine-deficient areas have been advanced. These include the use of foods known to be rich in iodine, iodinization of water supplies, administration of iodine at regular intervals in the form of solutions or tablets, the general use of iodized salt, and the consumption of iodized milk (Fig. 2).

The use of iodized salt has thus far proved the most widely adopted method. The nearly universal employment of common salt for seasoning and cooking, as well as the ready preparation and low cost of iodized salt, makes this the popular method. The use of milk with an increased iodine content has also been suggested as suitable, especially for children who ordinarily consume large quantities. Effective iodine prophylaxis should conform to local conditions, since no single method will reach all those persons who need iodine.

The iodized salt originally recommended in 1924 by the Michigan State Medical Society in conjunction with the State Board of Health, and subsequently employed in Michigan with such outstanding results, originally contained 0.02% of sodium iodide (17). However, after careful consideration, the Goiter Study Committee of the American Public Health Association reached the conclusion that the addition of 0.01% of potassium iodide plus a stabilizer should be sufficient. The importance of a stabilizer was emphasized in view of previous experience that iodine may be lost from iodized salt and thus impart a yellowness and a halogen odor on liberation of elemental iodine.

It has been estimated that the average adult ingests about 6.2 Gm. of salt daily. Calculated on this basis, the approximate amount of potassium iodide intake would be 620 μg., which is equivalent to about 474 μg. of iodine. This is more than twice the amount we have suggested as optimal and would amply provide a person with a sufficient reserve (17).

TABLE III[a].—THE IODINE BALANCE IN DISEASES OF THE THYROID GLAND

(A Comparison of the Iodine Balance of Normal Individuals with that of Nodular and Exophthalmic Goiter Patients)

Type of Goiter Present	No. of Patients	Total Days of Investigation	Average BMR, %	Average Blood Iodine μg. %	Average Output per 3-Day Period — Urine, μg.	Feces, μg.	Sweat, μg.	Total, μg.	Average Intake per 3-Day Period, μg.	Average Balance per 3-Day Period, μg.
1. None–normal controls	3	24	Minus 7	4.3	154	31	28	213	87	−126
2. Nontoxic nodular	2	18	Minus 8	3.0	120	31	29	180	74	−106
3. Toxic nodular	2	15	Plus 28	8.5	323	149	38	510	117	−393
4. Exophthalmic	3	33	Plus 40	9.0	204	164	46	414	86	−328

[a] Note the increased negative balance in patients with hyperthyroidism, also the increased BMR and blood iodine levels (14).

THE CIRCULATING THYROID HORMONE

Another problem in which an adequate micromethod of determining biologic iodine has proved of real clinical value is in the estimation of the amount of circulating, iodine-containing, thyroid hormone. The nature and amount of the blood iodine has thus become a useful clinical index of thyroid function. Thus, in patients with toxic nodular and exophthalmic goiter, the whole-blood iodine (18), along with the basal metabolic rate (19), as well as the excretory iodine (15), are significantly higher than normal (Table III). It is important to know how much of this blood iodine is directly resultant to the intake iodine of nutrition (Table II), to the various diagnostic procedures which may have been applied and to any previous therapy; as well as how much is dependent on the daily formation of thyroid hormone and its breakdown products. As a consequence, various fractionation methods have been devised to determine which portion of the blood iodine most nearly coincides with the "*circulating thyroid hormone.*" One of the early problems encountered was to separate the iodine bound to the proteins of the blood from the more soluble "inorganic" fraction. Even more precise steps of delineation have been developed by Salter. Nevertheless, with the simpler clinical methods now available (29), the differential diagnosis of thyroid disease has become more precise. It is now possible actually to differentiate hyperthyroidism from hypermetabolism (30).

After an extensive series of studies, it became evident that in central Ohio the average normal basal metabolic rate was minus 5 ± 8% (19) and the corresponding *whole-blood* iodine 4.2 ± 1.2 μg. %. The average normal protein-bound iodine of whole-blood (acetone-insoluble) was 0.9 ± 0.2 μg. % (20). This fraction is significantly decreased in patients with hypothyroidism (20). Helpful clinical progress has recently been made in recognizing the early development of hyperthyroidism by correlating the protein-bound blood iodine with the basal metabolic rate as well as the symptoms ordinarily suggestive of hyperthyroidism (30).

Thus, in 178 patients with *uncomplicated* nontoxic nodular goiter, it was found that the protein-bound blood iodine increases progressively from 0.51 μg. % in those with basal metabolic rates below minus 21% to 1.32 μg. % in those exhibiting basal metabolic rates above plus 11% (21). A significant linear relation between the basal metabolic rate and the protein-bound blood iodine was thus established in uncomplicated instances of this disease. Moreover, of the 66.8% of these patients exhibiting hyperthyroid-like symptoms, 60% had elevations of the PBI[2] and only 47% elevation of the BMR, thus demonstrating that the protein-bound blood iodine is a better index of thyroid function than the basal metabolic rate alone (21, 22). However, both taken together are most helpful to the clinician.

The protein-bound blood iodine has thus become an important aid in the differential diagnosis of those diseases which may mimic hyperthyroidism. These include hypertension, organic heart disease, "neurocirculatory asthenia," anxiety state, psychoneuroses,

[2] The protein-bound blood iodine.

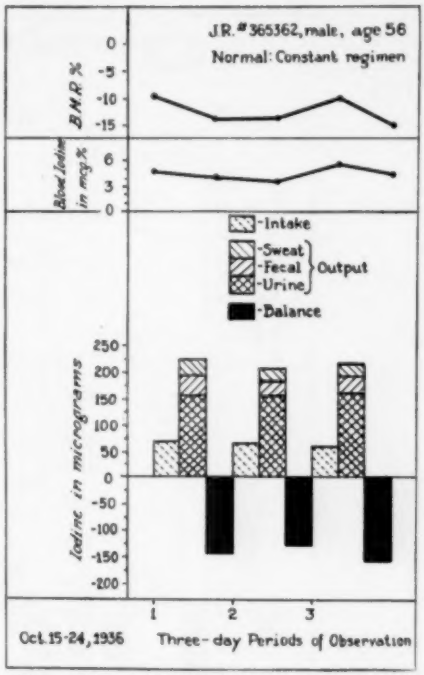

Fig. 1.—Normal iodine balance. Note that the intake is purposely kept low; the urinary excretion is high and the balance consequently negative (14).

neoplastic diseases, and certain endocrine disturbances, such as acromegaly and the adreno-cortical syndromes. In hyperthyroidism, the protein-bound blood iodine is increased; however, in the nonhyperthyroid hypermetabolic states the protein-bound blood iodine is normal or may be even subnormal unless hyperthyroidism is also present (30).

RADIOACTIVE IODINE

Within the decade preceding the development of "atoms" to be used for destruction, iodine, and few elements even before it, had been going "atomic," yet with a scientific intent then purely constructive. Thus, it had been found that these radioactive or "tagged" atoms could be employed to determine the ultimate fate of ingested substances in the body, as well as their intermediate history of absorption, distribution, synthesis, and eventual excretion. Prior to the use of these "*radioactive isotopes*" there was no method available for differentiating the iodine taken during a given experiment from that already present in the human body and particularly in the thyroid gland. Radioactive iodine, in the truly minute quantities in which it could be employed, lent itself readily to the determination of the fate of intake iodine as well as to the process of the succeeding endogenous iodine metabolism. Studies using radioactive iodine (I^{131}) soon demonstrated that iodine was quickly brought to the

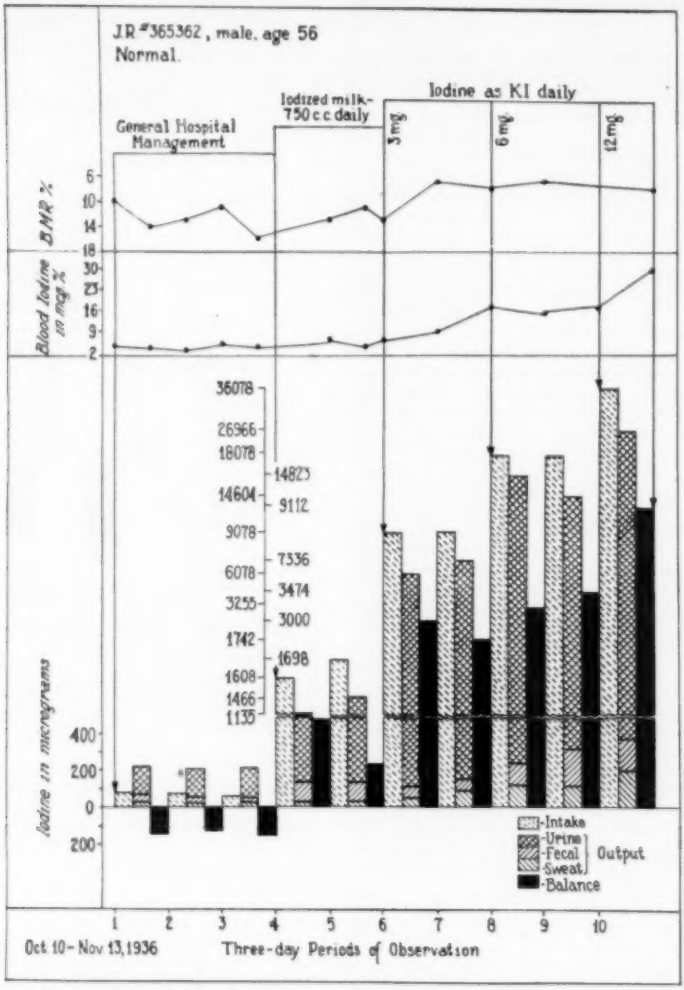

Fig. 2.—The effect of increased iodine intake on the iodine balance. Note the negative balance on a low intake and the storage resultant to adding supplemental iodine (14).

thyroid gland by the blood stream and there rapidly converted by the thyroid cells into diiodotyrosine and eventually thyroxine, the thyroid hormone (Fig. 5), moreover, that within a matter of a few hours these organic iodine compounds returned to the circulation (23–25).

From a consideration of normal thyroid activity, using the *"tracer"* method, was but a step to a more penetrating investigation of iodine metabolism in thyroid disease. "Labeled" iodine, usually I^{131}, was used to study the pathologic thyroids of experimental animals. Subsequently the metabolism of radioiodine in human thyroids, later removed at operation, was followed, particularly by *"autographs"* (26, 28) (Fig. 5).

An investigation of the metabolism of radioactive iodine used as a "tracer" in studying six nodular goiters revealed that the nodular formations, and particularly one nodule of a fetal adenomatous type, were less active in their uptake than the surrounding thyroid tissue (24) (Fig. 5). This was demonstrated by the lesser iodine content in the nodules than in the paranodular tissues, a smaller radioiodine fixation and a slower turnover of iodine. Moreover, the formation of thyroxine, isolated chemically and determined by the "tracer" technique, was comparatively slight in the nodular tissue(Fig.5). These studies were conducted chiefly on nontoxic nodular goiters. A study of toxic nodular goiter revealed that the paranodular tissue rather than

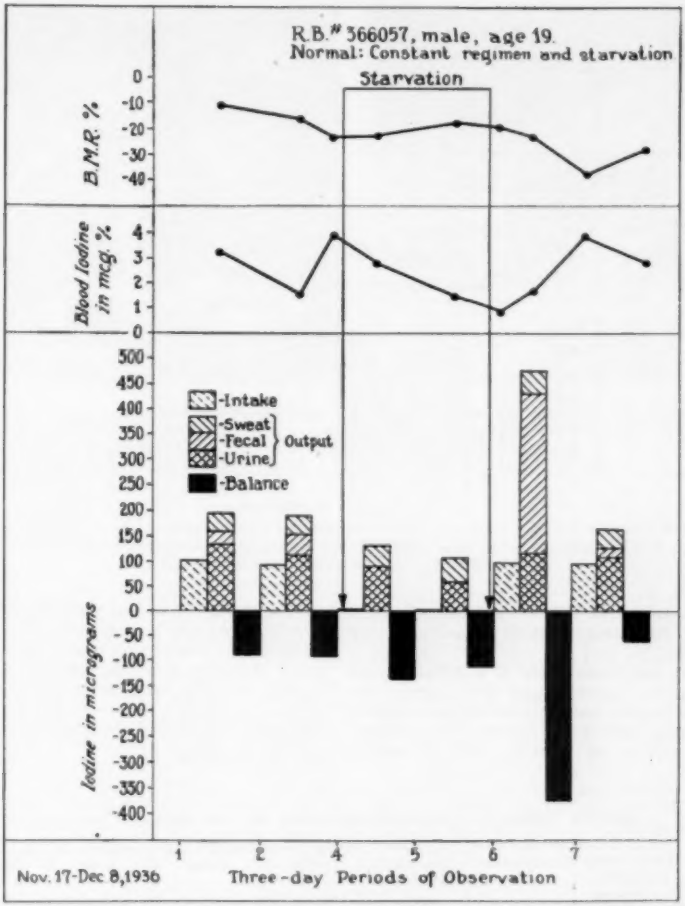

Fig. 3.—Iodine excretion continues during starvation. Note the increased fecal loss during the recovery period (14).

the nodules themselves forms the greater part of the "toxic" secretion (32).

The behavior of the nodular activity revealed that local factors by themselves may affect the iodine metabolism within the thyroid gland. In addition, other influences were demonstrated. Thus, a high level of the blood iodine "saturating" the gland with iodine may result in an increased storage of thyroxine inside the colloid (24). Moreover, that so-important pituitary factor—the thyrotropic hormone—when excessive or deficient, may activate or slow down the iodine turnover (25). The specific activity of the diiodotyrosine fraction has been found intermediary between that in the inorganic fraction and that in thyroxine, at least during the first forty-eight hours, thus possibly indicating that thyroxine is synthesized more slowly than diiodotyrosine, its presumable precursor (24).

The histologic method, or "*autograph*" technique, by utilizing the radiation effect of radioactive iodine on the photographic plate has made it possible to trace systematically the various states of the deposition of iodine in the thyroid cell. A comparison of the chemical, biophysical, and histologic results reveals that radioiodine passes through the cellular epithelium of the thyroid follicles rather rapidly, to be stored eventually as diiodotyrosine and thyroxine within the thyroid colloid.

A preferential distribution of radioactive iodine has been demonstrated in the colloid of normal rats (26), as well as in pituitary-treated and hypophysectomized animals (27). It can now be similarly demonstrated in the human thyroid gland, from studies made on surgically removed goiters. Microscopic sections of the diffuse hyperplastic goiter of Graves' disease removed fifteen hours after the administration of radioiodine demonstrate this clearly (28) (Fig. 6). *The storage of radioiodine within the colloid* is noteworthy. The radioiodine has actually photographed itself, a manifestation of

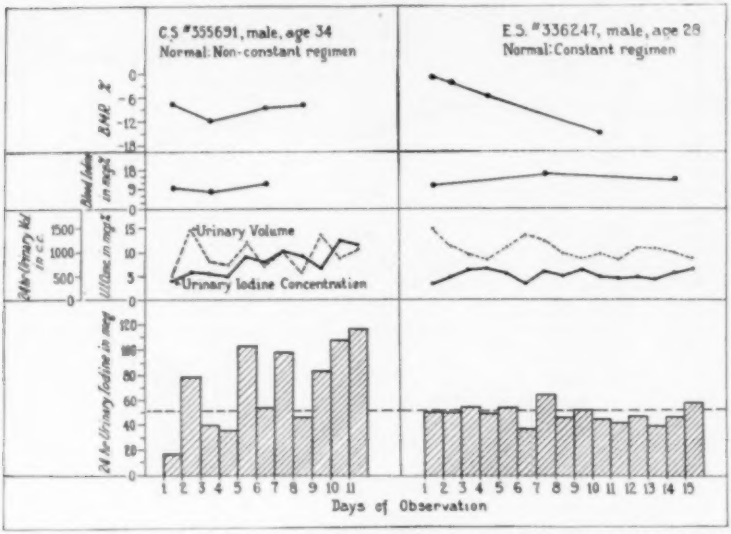

Fig. 4.—The urinary iodine loss. Note the variability on an ordinary diet (C.S.) and the constancy (E.S.) on a monotonous diet (15).

the atomic energy of I^{131}. It is localized within the colloid, its passage through the cell was only transient (Fig. 6).

A similar study was made of a diffuse colloid goiter (Fig. 5). Again, in any given section, the radioiodine localizes preferentially within the colloid (28). Further comparative study of the different types, as well as the different parts of nodular goiters, should prove even more revealing. *Struma nodosa trabecularis* is embryonic in character, and quite cellular as well, with little or no colloid formation. It should, therefore, take up but a minimal amount of iodine, since iodine is stored essentially within the colloid. On the other hand, with *struma nodosa microfollicularis* one should usually obtain a significant "autograph."

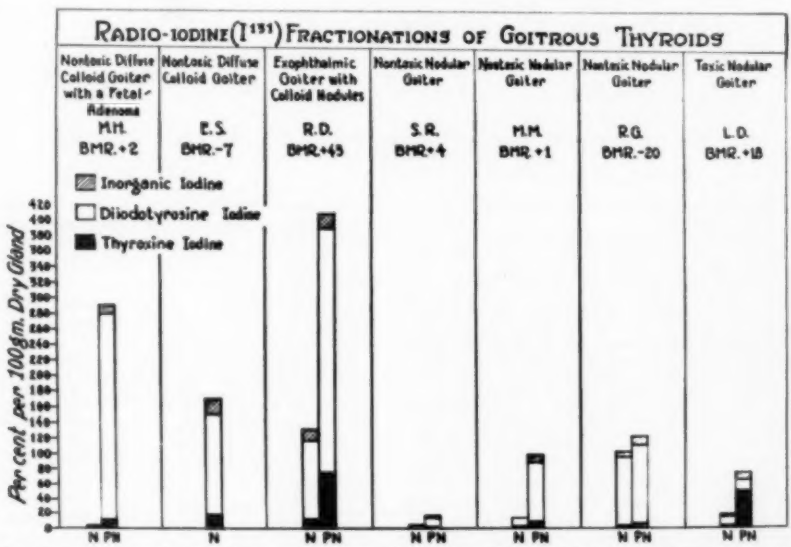

Fig. 5.—The use of radioiodine as a "tracer." Note the variability of hormone formation in the different types of goiter (32).

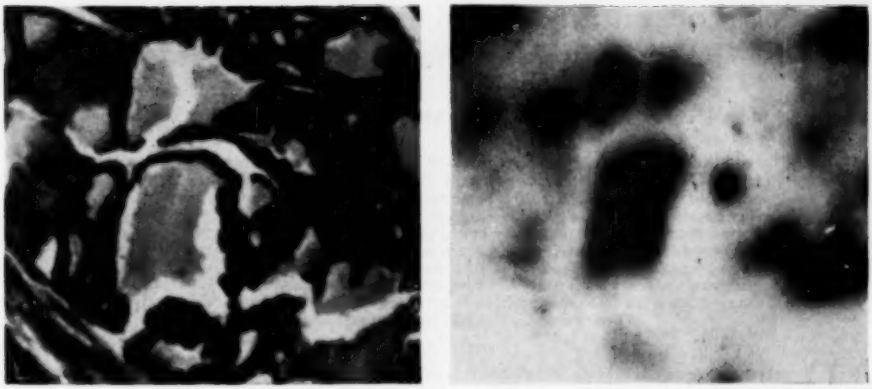

Fig. 6.—The "autograph" effect of radioiodine. The figure at the left is the stained histologic section of the goiter; at the right is its "autograph." Note that fifteen hours after administration the radioiodine has passed through the thyroid cells and is stored in the colloid substance (28).

The use of radioiodine, usually I^{131}, as a "tracer" under these conditions of varying thyroid disease demonstrates the rate of iodine storage within the thyroid, its rate of synthesis into diiodotyrosine and eventually thyroxine, as well as the subsequent mobilization, circulation, and elimination of these substances and their iodine-containing by-products. Its use as an "autograph" enables a "follow-up" of its quantitative distribution into the various tissues. Radioiodine has more recently taken its place in the therapeutic management of certain forms of thyroid disease and particularly thyroid cancer. Thus, as iodine goes "atomic," our horizons widen, our facilities for learning become greatly augmented, new facts result in new ideas, and our hope for progress in the control of disease is again stimulated.

This autograph (Fig. 6) is prophetic. Bichat demonstrated the significance of the microscopic structure of the various tissues. And Virchow taught us the significance of those changes which take place when disease enters and changes the normal histologic picture. Yet here we see the beginning of new microscopic science, demonstrated by "autographs," which clearly is to follow. For in this we will learn more of histochemistry and the distribution of the various chemical substances so important to human metabolic processes, within the living cells and tissues.

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The Concentration and Purification of Diphtheria Toxoid by Polyphosphate Precipitation*

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Diphtheria toxoid can be concentrated and purified by the use of a polyphosphate and acid in a simple and convenient manner (1). A polyphosphate solution and 1 *N* acid are added to the crude bacterial filtrate. The toxoid is precipitated, leaving nearly all the nonspecific nitrogen in solution. This precipitate may be separated from the supernatant by centrifugation and decantation. It is subsequently dissolved in 3 per cent sodium citrate solution. This solution may be standardized as to Lf. content and used for the preparation of purified fluid toxoid, polyantigens, and alum-precipitated toxoid. The method is rapid and inexpensive. It can be carried out with equipment which is generally available in bacteriological, chemical, pharmaceutical, and immunochemical laboratories.

DESPITE the many methods which have been proposed for the purification and concentration of diphtheria toxoid, none has achieved widespread acceptance and crude detoxified bacterial filtrates are still employed prophylactically. There are two principal reasons for this lack of acceptance of such methods. First, the methods of purification proposed are generally time-consuming, and the losses are large, so that the processes are economically unfeasible and secondly, the equipment required for certain methods is relatively so expensive as to preclude their general use.

A survey of the various methods which have been proposed for the purification and concentration of diphtheria toxoid and toxin discloses that these methods may be grouped for convenience into two major categories. These are, first, chemical methods, that is precipitation of the toxin or toxoid by protein precipitants, with subsequent solution of the separated toxin or toxoid in buffered mixtures; and secondly, physical methods such as adsorption of the active material on an adsorbent with subsequent elution of the toxoid or toxin, or absorption of impurities on an absorbent such as activated carbon, or ultrafiltration.

Solvent precipitation was investigated by Schmidt, Hansen, and Kjaer (2) who experimented with methyl and ethyl alcohol and acetone at various temperatures as low as −10° and even earlier Brieger and his co-workers had used alcohol for the precipitation of diphtheria toxin. Pillemer and co-workers (3) studied the use of alcohols as precipitants for diphtheria toxoid exhaustively, and Pillemer's variation has been adopted as the method of choice by some investigators. This method, however, requires several precipitations at temperatures not higher than −5°, facilities for storage of the toxoid at this temperature, and refrigerated centrifuges. In addition the method is scarcely less laborious than that of Pappenheimer (4).

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METHOD

Diphtheria toxoid prepared from toxin obtained with Park-Williams No. 8 strain of *Corynebacterium diphtheriae* maintained on Bacto Veal No. 2 broth was precipitated by the addition of a solution of sodium salt of a polyphosphate and 1 *N* acid. The sample was then permitted to stand until a flocculent precipitate formed. The precipitate and supernatant liquid were then transferred to centrifuge cups and centrifuged to separate the precipitate from the supernatant. The supernatant phase may be decanted off readily. The precipitate was dissolved in a 3% sodium citrate solution. In this form the concentrate may be stored until it is desired for use, the addition of Merthiolate 1:10,000 being useful for preservation.

PROCEDURE

To 640 ml. of the diphtheria toxoid, 64 ml. of the 10% sodium hexametaphosphate was added. After mixing well with a glass stirring rod, 72 ml. of the *N* sulfuric acid was added. An immediate turbidity was noticed. When the mixture was allowed to stand for forty-five minutes at room temperature or for twenty to thirty minutes at 43°, the turbidity was resolved into a flocculent precipitate. The precipitate was gently mixed with the supernatant liquid and this mixture was transferred to 250-ml. centrifuge cups. The centrifuge was run at 2,775 r.p.m. for five minutes. After this time the cups were removed. The precipitate was found packed hard and the supernatant clear. The supernatant liquid was decanted off and the centrifuge cups inverted on filter paper and allowed to drain for five or ten minutes. Three milliliters of the 3% sodium citrate solution was then pipetted into one of the centrifuge cups and the mixture was triturated with a clean smooth glass rod until the solid material was dissolved. This solution was then transferred to a second centrifuge cup. The first centrifuge cup was washed with 2 ml. more of the 3%

sodium citrate solution which was added to the material in the second cup. From there the material was transferred to a graduate and all the cups given a final washing with 2–5 ml. of the 3% sodium citrate solution, the amount depending upon what was necessary to give a total final volume of 20 ml. The sample was transferred to a small stoppered bottle or tube and stored.

RESULTS

The concentrated diphtheria toxoid produced by the above procedure was tested by the Ramon flocculation test. The purity of the product is given in terms of the Lf./mg. of nitrogen. The nitrogen determinations were made by one of the authors' modifications of the Pregl-Parnas-Wagner method for the micro determination of nitrogen (5, 6).

TABLE I.—THE CONCENTRATION OF DIPHTHERIA TOXOID PRODUCED UPON NATURAL MEDIA

Sample	Lf./Ml.	Kf.	Yield, %
Original	40	60 min.	...
I-E	1,044	4 hr.	81.5
I-F	1,280	6 hr. 10 min.	100.0
I-I 1	1,376	1 hr. 40 min.	100.0
I-I 2	610	2 hr. 15 min.	88.6
I-I 3	688	2 hr. 10 min.	100.0
I-J	1,376	2 hr. 45 min.	100.0

The purification factor may be defined as the number of times the Lf. concentration of a purified toxin or toxoid has been elevated over that of a parent or crude toxin or toxoid from which it had been prepared. The purification factor can be calculated by means of the formula:

$$\text{Purification Factor} = \frac{\text{Lf./mg. N in Purified Toxoid}}{\text{Lf./mg. N in Parent Toxoid}}$$

With the aid of this formula, the purification factors given in Tables II and IV were calculated. Also listed in Tables II and IV is the per cent of nitrogen removed by the purification and concentration method employed in this work. In order to calculate this figure, the mg. of nitrogen per ml. of a given parent toxoid is multiplied by the number of ml. used in an experiment. This result is the total amount of nitrogen present. The mg. of nitrogen per ml. found for a given concentrated toxoid is multiplied by the volume of concentrate recovered. This result is the total amount of nitrogen present in the concentrated toxoid. Dividing the second value by the first and multiplying by 100 yields the per cent of nitrogen remaining in the concentrated toxoid. Subtracting this result from 100 gives the per cent of nitrogen removed.

The crude bacterial filtrate produced on natural media contained approximately 40 Lf./ml. and about 10 Lf./mg. of nitrogen. The least concentrated of the treated material contained 610 Lf. units per ml. and the least purified of the treated material contained 362 Lf./mg. of nitrogen. Most of the material produced is considerably better than this. Both the products just mentioned resulted from variations on the most successful procedure which was detailed previously. An example of the material produced by the normal procedure is a product containing 1376 Lf. units per ml. and 548 Lf./mg. of nitrogen. The product just mentioned was concentrated 32 times as a result of a single precipitation and has a purification factor of 54.8. There was no loss of material involved in the procedure since the product was recovered in 100% yield. The average purification factor was 48.4. This figure includes samples produced by variations in the method, as does the figure for the average yield, which is 95.9.

TABLE II.—THE PURIFICATION OF DIPHTHERIA TOXOID PRODUCED UPON NATURAL MEDIA

Sample	Mg. N/Ml.	Lf./Mg. of N	Purification Factor	N Removed, %
Original	4.35	10.0	...	...
I-E	2.29	456	45.6	98.36
I-F	2.605	492	49.2	98.13
I-I 1	2.51	548	54.8	98.20
I-I 2	1.45	421	42.1	97.92
I-I 3	1.90	362	36.2	97.27

TABLE III.—THE CONCENTRATION OF DIPHTHERIA TOXOID PRODUCED UPON SEMISYNTHETIC MEDIA

Sample	Lf./Ml.	Kf., Min.	Yield, %
Original	72.6	15	...
I-K 1	1,655	15	100
I-K 2	2,333	15	100
I-K 3	1,655	15	100
I-K 4	1,655	25	100
I-L	2,333	15	100

TABLE IV.—THE PURIFICATION OF DIPHTHERIA TOXOID PRODUCED UPON SEMISYNTHETIC MEDIA

Sample	Mg. N/Ml.	Lf./Mg. of N	Purification Factor	N Removed, %
Original	3.06	23.7	...	...
I-K 1	1.91	866	36.6	97.26
I-K 2	1.94	1,200	50.7	98.02
I-K 3	1.62	1,021	42.2	97.69
I-K 4	2.05	807	34.1	97.08
I-L	1.95	1,194	50.4	98.01

When diphtheria toxoid produced upon the semisynthetic media of Mueller and Miller (7) was used, an average of 1,962 Lf./ml. was obtained. The average purification factor was 42.8. The average Lf./mg. of nitrogen obtained in one precipitation was 1,018, which is almost 50% purity if 2,146 Lf./mg. of nitrogen is accepted as the figure for 100% purity (4).

Various other polyphosphates, such as sodium tripolyphosphate, tetrasodium pyrophosphate, and sodium tetraphosphate, in addition to sodium hexametaphosphate, were found applicable to the precipitation of the toxoid produced on semisynthetic media. The flocculation time of this material was not increased appreciably over that of the original crude bacterial filtrate and all products were recovered in 100% yield.

The crude bacterial filtrate from which the above products were obtained, contained 72.6 Lf./ml. and 23.7 Lf./mg. of nitrogen. The Kf. of both crude material and purified products was about fifteen minutes.

DISCUSSION

The polyphosphate-acid method for the concentration and purification of diphtheria toxoid was chosen because of its ease, simplicity, and adaptability. Pillemer's modification (8) of the ethyl and methyl alcohol method of Schmidt, Hansen, and Kjaer (2) is recommended by some authorities, but the solvent method of precipitation has serious disadvantages which are inherent in the character of the materials used. The most serious is that the heat of solution of the alcohol in water is so high that it becomes necessary to use very low temperatures of the order of −20° for precipitation and centrifugation. As previously mentioned this requires elaborate equipment which is not generally available.

The principal purposes in the concentration and purification of diphtheria toxoid are: (*a*) to provide a more potent toxoid; (*b*) to enable it to be used in polyantigen preparations without increasing the volume which is usually injected; and (*c*) to eliminate or minimize the reactions produced when crude preparations are used. Considering that these objectives are acheived by the products of the method outlined, and comparing this method with a number of those suggested, its advantages may readily be seen. The method is uncomplicated; the materials and equipment are inexpensive and readily procurable; the recovery ranges from 81 to 100% when estimated by the Ramon flocculation values; and over 97% of the nonspecific protein nitrogen is eliminated in one precipitation. Although the Kf., or flocculation time, is somewhat increased over that of the original material with toxoid produced on natural media, it has been noted specifically by one of the authors (9) in his paper on the concentration and purification of tetanus toxoid by the trichloroacetic acid method that this does not indicate any impairment of the antigenicity. An increase in flocculation time over that of the crude parent toxoid upon purification has been noted by many investigators, among whom are Ramon, Boivin, and Richou (10), and Ando and Komiyama (11). It may also be noted here that a decrease in flocculation time does not indicate an increase in the antigenicity, as evidenced by the poor protective power for mice of Pillemer's product. This product had a very rapid flocculation time, but the highest percentage protection obtained with it was 58%. In some cases the percentage protection dropped to 10%, which was a lower value than that recorded for the crude original material.

It is, however, with respect to flocculation time that this method has its greatest advantage over the Jacobs variation (9) of the Boivin and Izard trichloroacetic acid method (12). According to unpublished data the increase in the flocculation time caused by the polyphosphate acid method is far less than that caused by the trichloroacetic acid method. When a toxoid produced upon semisynthetic media is used there is no increase in the flocculation time upon concentration and purification with this method.

An important advantage of this method is that it gives purified toxoid in a concentrated form which may be readily stored in a small space, eliminating the necessity for large refrigerated storage facilities which are expensive to install and maintain.

Our tests show that this method is satisfactory for tetanus toxoid. The tetanus toxoid prepared upon semisynthetic media precipitates very rapidly at room temperature.

SUMMARY

Diphtheria toxoid can be concentrated and purified by a method employing a polyphosphoric acid as a precipitant. The toxoid is mixed with the polyphosphate and acid which forms a polyphosphoric acid and is precipitated from the crude bacterial filtrate. The precipitate is recovered by centrifugation and is dissolved in a buffer. Recovery ranges from 81 to 100% and over 97% of the nonspecific protein nitrogen is eliminated in one precipitation without washing. Flocculation time is only moderately increased in most cases. It is not increased at all when a toxoid produced upon semisynthetic media is used. The method is rapid, inexpensive, and needs only such equipment as is commonly found in immunochemical, chemical, and bacteriological laboratories.

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Urinary Excretion of the Metabolic Products of Parathion Following Its Intravenous Injection*,†

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A series of experiments designed to identify and estimate metabolic products excreted in the urine of dogs following intravenous administration of parathion is described and the results reported. The principal urinary excretion product is believed to be *p*-nitrophenol. For the determination of this excretion product it was reduced to *p*-aminophenol and determined colorimetrically by an established procedure. During the course of the studies plasma cholinesterase levels were significantly lowered following the administration of parathion in doses of 1 mg./Kg. or more.

SEARCH of the literature reveals very little concerning the metabolic fate of parathion (O,O-diethyl O,*p*-nitrophenyl thiophosphate) in the mammalian body, although toxicity and cholinesterase inhibitory action have received considerable attention (1–3). Hazleton (2) and Lehman (4) reported the presence of parathion in the urine following administration of toxic doses. They further found that parathion, when administered in toxic doses, was not stored in the body. Both authors employed the colorimetric method of Averell and Norris for parathion (5).

An examination of the parathion molecule suggests the possibility that parathion might be split in the body to *para*-nitrophenol and O,O-diethyl thiophosphate. Mountain, *et al.* (6), suggest such an enzymatic hydrolysis.

The metabolism of *para*-nitrophenol as reviewed by Williams (7) appears to involve detoxification by (*a*) conjugation with glycuronic acid or sulfates, or (*b*) acetylation. The nitro-radical is progressively reduced through *para*-nitrosophenol, hydroxyphenylhydroxylamine to *para*-aminophenol. The same mechanisms may exist with the intact parathion molecule.

Recently Pankaskie, *et al.* (8), reported that following chronic feeding of parathion to cows, *para*-aminophenol was found in the urine; however, the amount of *para*-aminophenol present represented but about 10 per cent of the total parathion fed. Their analytical method was based on the indophenol blue color as modified by Brodie and Axelrod (9). They did not detect *para*-nitrophenol by the alkaline yellow colorimetric method used for its identification.

Mountain, *et al.*, in a recent publication (6) described a new technique for recovery of added *para*-nitrophenol from urine. This method was developed pursuant to their investigation of parathion metabolism and appears to yield better recovery than the Brodie and Axelrod (9) method.

The present report summarizes a series of experiments directed at the possible identification and estimation of certain metabolic products excreted in the urine following intravenous administration of parathion.

METHODS

Plasma cholinesterase activity was determined by the titrimetric method of Stedman, *et al.* (10).

Total *p*-aminophenol, i.e., both free and conjugated, was determined by the colorimetric method of Brodie and Axelrod (9). Aggregate *p*-aminophenol, i.e., the free and conjugated forms of *p*-nitrophenol, *p*-nitrosophenol, hydroxyphenylhydroxylamine and *p*-aminophenol was determined by reduction of acid hydrolyzed urine and estimating as *p*-aminophenol.

Procedure.—One to five milliliters of the urine samples are pipetted into a glass ampul and the volume adjusted to 5 ml. with distilled water, if necessary. One ml. of concentrated hydrochloric acid is added, the ampul sealed and placed in a boiling water bath for three hours. An alternative procedure, as recommended by Brodie and Axelrod (9), is to place the acidified urine mixture in a regular test tube and hydrolyze it in an autoclave for ninety minutes at 15 lb. pressure.

After hydrolysis the specimen is reduced as follows: The contents of the ampul are transferred quantitatively into a test tube and 1 Gm. of zinc dust is added. After cessation of the evolution of gas, the test tube is placed in a boiling water bath for ten minutes. The contents of the test tube are then cooled to room temperature, filtered and volume adjusted to 6 ml. with distilled water. A 5-ml. aliquot of this reduced hydrolysate is then extracted with an ether-isoamyl alcohol mixture as recommended by Brodie and Axelrod and their procedure followed for the estimation of total *p*-aminophenol (9). The density of the indophenol

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TABLE I.—ANALYSIS OF DOG URINE FOLLOWING INTRAVENOUS ADMINISTRATION OF *p*-AMINOPHENOL 4 MG./KG. URINE WAS ANALYZED FOR TOTAL *p*-AMINOPHENOL AND AGGREGATE *p*-AMINOPHENOL

Time, Hr.	Symptoms	Total *p*-Aminophenol, μg./Cc.	Aggregate *p*-Aminophenol, μg./Cc.	Total *p*-Aminophenol μg./Specimen	Aggregate *p*-Aminophenol, μg./Specimen	% Recovery of Administered *p*-Aminophenol
0	None	0	0	0	0	0
1	None	6.3	5.8	541.2	513.1	1.5
2	None	8.2	8.2	853.8	853.8	2.5
4	None	16.2	15.3	761.4	719.1	2.1
6	None	12.2	12.0	573.4	565.4	1.6
8	None	9.8	9.8	607.6	607.7	1.8
8–24	None	11.2	11.2	876.7	876.7	2.5
					Total recovery	12.0

blue color developed was measured at 630 mμ by means of the Beckman Model DU Spectrophotometer. A standard reference curve was employed in calculating the concentration of *p*-aminophenol present.

EXPERIMENTAL

Preliminary experiments in dogs established the fact that following the intravenous injection of *p*-aminophenol, its excretion in the urine was slow but continued almost uniformly throughout the first twenty-four hours (Table I). Following intravenous administration of *p*-nitrophenol the recovery as *p*-aminophenol, without reduction, was negligible during the first two hours (Table II). An appreciable quantity was recovered during the third hour after administration. Although the urine exhibited the alkaline yellow color of *p*-nitrophenol, this method of analysis is nonspecific and subject to interference by urinary pigments.

In a female mongrel dog, parathion, 10 mg./Kg., was injected intravenously as an alcoholic solution containing 200 mg./cc. Urine was collected by catheterization at hourly intervals for the first eight hours and analyzed for *p*-aminophenol after hydrolysis, but without reduction. The results, summarized in Table III, indicate that although *p*-aminophenol is excreted under these conditions, the recovery in terms of parathion is relatively small,

TABLE II.—ANALYSIS OF DOG URINE FOR TOTAL *p*-AMINOPHENOL FOLLOWING INTRAVENOUS ADMINISTRATION OF *p*-NITROPHENOL, 5 MG./KG.

Time, Hr.	Symptoms	Total *p*-Aminophenol, μg./Cc.	Total *p*-Aminophenol, μg./Specimen	% Recovery of Administered *p*-Aminophenol
0	None	0	0	0
1	None	6.4	70.4	0.2
2	None	1.1	7.2	0.02
3	None	24.6	425.8	1.0
4	None	2.7	39.0	0.09
5	None	2.0	29.0	0.06
6	None	1.5	64.4	0.15
7	None	1.3	39.0	0.09
8	None	1.3	23.8	0.05

as was the case with injected *p*-aminophenol. For comparison with later experiments the severity of the intoxication in this dog should be noted.

From these experiments it is apparent that without reduction of the hydrolyzed urine, the recovery of administered parathion would not be sufficient to permit detection of small doses and provide for adequate quantitation. To determine the efficiency of extraction and of the reduction process, eleven solutions, containing *p*-nitrophenol, *p*-aminophenol and various ratios of these, were prepared in dog urine. Analysis of these solutions, with and without

TABLE III.—ANALYSIS OF DOG URINE FOR TOTAL *p*-AMINOPHENOL FOLLOWING INTRAVENOUS ADMINISTRATION OF PARATHION, 10 MG./KG.

Time, Hr.	Symptoms	Total *p*-Aminophenol, μg./Cc.	Total *p*-Aminophenol, μg./Specimen	% Recovery Calculated as Parathion
0	None	0	0	0
1	Tremors Salivation	25.6	141.0	0.4
2	Tremors	22.9	91.6	0.3
3	Tremors	15.1	83.1	0.2
4	Diarrhea	15.9	79.8	0.2
5	Tremors Vomiting Diarrhea	10.6	47.7	0.1
6	Lassitude	4.2	23.1	0.08
7	Lassitude	3.7	16.7	0.05
8	Lassitude	2.1	10.6	0.03
8–24	Bloody diarrhea Respiration shallow Heart beat imperceptible Condition very poor	3.2	44.8	0.1

TABLE IV.—ANALYSES OF URINE FOLLOWING INTRAVENOUS ADMINISTRATION OF *p*-NITROPHENOL, 4.0 MG./KG. URINE WAS ANALYZED FOR TOTAL *p*-AMINOPHENOL AND AGGREGATE *p*-AMINOPHENOL

Time, Hr.	Symptoms	Total *p*-Aminophenol, μg./Cc.	Aggregate *p*-Aminophenol, μg./Cc.	Total *p*-Aminophenol, μg./Specimen	Aggregate *p*-Aminophenol, μg./Specimen	% Recovery of Administered *p*-Nitrophenol
0	None	0	0	0	0	0
1	None	0.8	49.3	57.5	3,648.2	15.0
2	None	0.9	16.3	72.0	1,322.9	5.4
4	None	0.8	12.7	64.0	1,055.8	4.4
6	None	0.8	5.0	52.1	332.0	1.3
6–24	None	1.5	2.4	106.6	173.7	0.8
					Total recovery	26.9

hydrolysis, indicated that (*a*), *p*-aminophenol could be recovered to the extent of approximately 70% in either case, (*b*) added *p*-nitrophenol could be recovered to the extent of approximately 57% after reduction, and (*c*) recovery of mixtures of the two (aggregate *p*-aminophenol) was between these two values. This recovery of *p*-nitrophenol and mixtures of *p*-nitrophenol and *p*-aminophenol, up to approximately the efficiency of the extraction process, appears to validate the reduction process to convert *p*-nitrophenol and intermediates to *p*-aminophenol in the determination of excreted metabolites following parathion administration.

The *in vivo* recovery of intravenously injected *p*-nitrophenol was determined following a 4 mg./Kg. dose. The urine was collected by catheterization and analyzed after hydrolysis for both total and aggregate *p*-aminophenol. The results, summarized in Table IV, confirm the observation that small amounts of *p*-aminophenol are excreted and that reduction converts the *p*-nitrophenol to *p*-aminophenol. The total recovery approximates 27% during the first twenty-four hours, the rate being highest during the first hour and gradually decreasing.

Based on the above experiments a series of dogs were subjected to progressively increasing doses of intravenously injected parathion. These dogs were carefully observed for symptoms of parathion intoxication. Cholinesterase activity in the plasma was determined at intervals and the urine obtained by catheterization was analyzed for both total and aggregate *p*-aminophenol. These results are summarized in Tables V through VIII. At a dose of 1 mg./Kg. there was an 88% inhibition of the plasma cholinesterase activity, there were no observable symptoms, and 21.3% of the administered parathion was recovered as aggregate *p*-aminophenol. At 2 mg./Kg. the dog was asymptomatic, although the plasma cholinesterase was totally inhibited. The rate of recovery of parathion as aggregate *p*-aminophenol was greatest during the first four hours and 50% of the administered parathion was recovered within twenty-four hours. Throughout all of these experiments the recovery as total *p*-aminophenol was relatively small, whereas after reduction there was as much as a 100-fold increase in recovery. At 8 mg./Kg. the plasma cholinesterase was totally inhibited and the animal showed severe symptoms characteristic of parathion intoxication. The ani-

TABLE V.—ANALYSES OF DOG BLOOD AND URINE FOLLOWING INTRAVENOUS ADMINISTRATION OF PARATHION, 1.0 MG./KG. BLOOD WAS ANALYZED FOR PER CENT INHIBITION OF PLASMA CHOLINESTERASE AND URINE FOR TOTAL *p*-AMINOPHENOL AND AGGREGATE *p*-AMINOPHENOL

Time, Hr.	Symptoms	% Inhibition Plasma Cholinesterase	Total *p*-Aminophenol, μg./Cc.	Aggregate *p*-Aminophenol, μg./Cc.	Total *p*-Aminophenol, μg./Specimen	Aggregate *p*-Aminophenol, μg./Specimen	% Recovery Calculated as Parathion
0	None	0	0	0	0	0	0
1	None	88	2.66	17.5	98.44	647.5	21.3
2	None	..	0	3.18	0	44.5	1.5

TABLE VI.—ANALYSES OF DOG BLOOD AND URINE FOLLOWING INTRAVENOUS ADMINISTRATION OF PARATHION, 2.0 MG./KG. BLOOD WAS ANALYZED FOR PER CENT INHIBITION OF PLASMA CHOLINESTERASE AND URINE[a] FOR TOTAL *p*-AMINOPHENOL AND AGGREGATE *p*-AMINOPHENOL

Time, Hr.	Symptoms	% Inhibition Plasma Cholinesterase	Total *p*-Aminophenol, μg./Cc.	Aggregate *p*-Aminophenol, μg./Cc.	Total *p*-Aminophenol, μg./Specimen	Aggregate *p*-Aminophenol, μg./Specimen	% Recovery Calculated as Parathion
0	None	0	0	0	0	0	0
1	None	85	3.0	109.1	30.0	1,091.0	13.6
2	None	100	2.0	69.0	36.0	1,242.0	15.5
4	None	100	0.9	6.2	157.5	1,076.3	13.4
6	None	92	0.6	1.3	108.0	232.8	2.9
8	None	94	0.9	1.1	73.1	94.6	1.2
8–24	None	94	0.4	1.4	70.0	245.0	3.1
24[a]	None	94	..	1.2	..	22.1	0.3
						Total recovery	50.0

[a] Urine collected by catheterization at end of 24 hours.

TABLE VII.—ANALYSES OF DOG BLOOD AND URINE FOLLOWING INTRAVENOUS ADMINISTRATION OF PARATHION, 4.0 MG./KG. BLOOD WAS ANALYZED FOR PER CENT INHIBITION OF PLASMA CHOLINESTERASE AND URINE FOR TOTAL *p*-AMINOPHENOL AND AGGREGATE *p*-AMINOPHENOL

Time, Hr.	Symptoms	% Inhibition Plasma Cholinesterase	Total *p*-Aminophenol, μg./Cc.	Aggregate *p*-Aminophenol, μg./Cc.	Total *p*-Aminophenol, μg./Specimen	Aggregate *p*-Aminophenol, μg./Specimen	% Recovery Calculated as Parathion
0	None	0	0	0	0	0	0
1	None	100	1.3	146.1	58.5	6,426.2	32.1
2	None	100	1.2	81.3	55.7	3,739.8	18.6
4	None	100	0.3	15.4	46.8	2,397.7	11.9
6	None	100	0.8	19.2	40.0	962.0	4.8
8	None	100	0.6	10.3	38.9	548.0	2.7
8–23	None	..	0.5	6.6	98.7	1,392.3	7.0
23–24	None	..	0.2	2.4	36.2	509.1	2.6
24[a]	None	100	0	2.0	0	225.4	1.1
						Total recovery	80.8

[a] Urine collected by catheterization at end of 24 hours.

TABLE VIII.—SYMPTOMS AND PER CENT INHIBITION OF PLASMA CHOLINESTERASE FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 8.0 MG./KG. OF PARATHION TO FEMALE DOG

Time, Hr.	Symptoms	% Inhibition Plasma Cholinesterase
0	None	0
1	Salivation Tremors	75
2	Tremors	100
4	Salivation Tremors	100
6	Tremors	100
8	Tremors	100
24	Lassitude	100

mal was anuric when catheterized one hour after injection and remained so during the entire twenty-eight hours of observation. The animal was then sacrificed while in a moribund condition and inspection of the urinary bladder revealed total collapse and anuria. On gross inspection of the transversely cut kidneys there was an obvious white striated area in the cortex. Microscopic inspection revealed the presence of a granular, coagulated material in the lumina of the tubules. When stained with Sudan IV, the descending limbs of Henley's loops showed numerous, varying-sized lipoid globules within the epithelial cells and densely packed, similar globules within the lumina. This was apparently producing an obstruction to the outlet of these tubules. In no other area did there appear to be any fatty changes within the fatty cells of the lumina.

In this dog, supportive treatment with atropine in doses of 1 mg./Kg. and metrazol in doses of 10 mg./Kg., administered as the condition suggested, failed to improve the condition.

DISCUSSION

Although the extraction of *p*-aminophenol from acid hydrolyzed and reduced urine by the ether-isoamylalcohol mixture is not complete, it is consistent throughout the range of *p*-aminophenol–*p*-nitrophenol mixtures studied. The excretion of *p*-nitrophenol following the administration of parathion appears to be more complete than when *p*-nitrophenol itself is injected and is proportional to the amount of parathion administered.

p-Nitrophenol appears to be a principal metabolic product of parathion, although traces of *p*-aminophenol are found. This being true, analysis for either of these materials would not be adequate, nor would they include the metabolic intermediates *p*-nitrosophenol and hydroxyphenylhydroxylamine. The procedure herein described converts *p*-nitrophenol and the intermediates to *p*-aminophenol which can be reliably determined in microgram quantities. Since *p*-nitrophenol constitutes approximately one-half of the parathion molecule, the conversion error is small. These factors render the method applicable to the small doses anticipated in parathion intoxication. The metabolic products can be readily detected in animals following doses of parathion which produce no symptoms other than cholinesterase inactivation.

SUMMARY

The principal urinary excretion product of parathion in mammals is probably *p*-nitrophenol.

p-Aminophenol is excreted in traces in the urine following parathion administration.

Reduction of *p*-nitrophenol and its metabolic intermediates to *p*-aminophenol provides a sensitive method of analysis.

In animals this method readily detects the metabolic products following administration of asymptomatic doses of parathion.

In all of the studies conducted, plasma cholinesterase levels were significantly lowered following parathion administration in doses of 1 mg./Kg. or more.

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The Relative Stability of Rutin and Quercetin in Alkaline Solution*

By EARL B. DECHENE

A study of the decomposition of rutin and quercetin in alkaline solution has been made, and it has been found that rutin is more stable than quercetin under these conditions.

THE METHOD reported (1) for the determination of rutin in tablets, when applied to tablets containing rutin and theophylline ethylenediamine, produced in some cases slightly lower values for rutin content than were expected. The alcoholic extracts of the tablets, obtained by this method, were found to have a value greater than *p*H 8.0. It was necessary to know, therefore, if rutin was decomposing under these conditions.

Link (2) describes the degradation of quercetin, the aglucone of rutin, by the action of alkali. No report of studies of the rate at which this degradation reaction occurs could be found in the literature. We studied, therefore, the rate of decomposition of both rutin and quercetin in aqueous-alcohol and alcoholic solutions of sodium hydroxide, ethylenediamine, and hydrochloric acid. The molar ratios selected for this study were of the same order as those present in the tablets.

The solutions of rutin or quercetin, containing various concentrations of the reactants, were refluxed or allowed to stand at room temperature, and the concentration of the flavonol in the solutions determined by the aluminum chloride color reaction (1). It was found that little or no decomposition of rutin occurred in aqueous-alcoholic solutions containing a molar concentration of slightly less than 2 moles of sodium hydroxide, or 30 moles of ethylenediamine per mole of rutin, and only slight decomposition occurred in solutions containing approximately 4 moles of sodium hydroxide per mole of rutin. Quercetin, under the same conditions, showed a rapid rate of decomposition in aqueous-alcoholic solutions containing a molar concentration of slightly less than 2 moles of sodium hydroxide or 30 moles of ethylenediamine per mole of quercetin. The slower rate of decomposition of rutin compared to quercetin in alkaline solutions suggested that the degradation of rutin occurs by reaction of the alkali on quercetin resulting from the hydrolysis of rutin, rather than by direct reaction on the flavonol glucoside. Considerable decomposition of rutin occurred in solutions containing a molar concentration of slightly less that 17 moles of sodium hydroxide or 60 moles of ethylenediamine per mole of rutin.

Paper chromatographic examination of the alkaline solutions of rutin showed no detectable amounts of quercetin. The absence of quercetin in the alkaline solutions in which the decomposition of rutin had occurred, indicated that quercetin decomposed as fast as it was produced by the hydrolysis of rutin. No decomposition of rutin occurred in aqueous hydrochloric acid solutions that were refluxed or allowed to stand at room temperature. Considerable amounts of quercetin could be detected on the paper chromatograms of these solutions, indicating that hydrolysis of rutin occurs readily in acid conditions.

The decomposition of rutin and quercetin in alkaline solutions varied with the concentration of alkali. For the reason above and because the concentration of hydroxyl ions present in the solutions would decrease throughout the course of the reaction by neutralization with the phenolic acids produced in the degradation of rutin and quercetin (2), the order of the reaction was not determined. The percentage of decomposition indicated the relative stability of rutin and quercetin in the alkaline solutions.

EXPERIMENTAL

Reagents

Rutin.—Rutin of N. F. IX quality and containing 2.6% quercetin was used without drying. Solutions in SDAG No. 1 G ethyl alcohol (85% v/v ethanol, 10% v/v methanol) were made to contain approximately 0.6 mg. of rutin per ml.

Quercetin.—A commercial grade of quercetin was used without drying, and a solution in SDAG No. 1 G ethyl alcohol was made to contain 0.3 mg. of quercetin per ml.

Ethylenediamine.—An aqueous solution was prepared containing 0.0195 Gm. of ethylenediamine per ml.

Procedure

The aluminum chloride color reaction previously described by the author (1) was used to determine

* Received May 28, 1951, from the Research and Control Laboratories of Charles E. Frosst & Co., Montreal, Canada.

TABLE I.—THE DECOMPOSITION OF RUTIN AND QUERCETIN IN 38% (v/v) OF AQUEOUS ALCOHOLIC SOLUTIONS AND ALCOHOLIC SOLUTIONS OF SODIUM HYDROXIDE, ETC

Expt. No.	Molar Concn. × 10^{-4} (Initial) NaOH	Rutin	Quercetin	HCl	Ethylene-diamine	Time, Hr.	Decompn., %	pH Initial	Temperature	Solution
1	6.15	3.612	..	..	..	4	0	8.3	Reflux	
2	6.15	3.822	..	..	..	3	10.25	8.3	Reflux	
						5.5	10.38			
						21	10.25			
3	6.15	3.612	..	..	..	48	4.7	8.3	Room	
						168	12.85			
4	12.30	3.60	..	..	..	2	9.7	9.7	Reflux	
5	12.30	3.60	..	..	..	20	21.4	9.7	Room	Alcohol 38%
6	63.10	3.822	..	..	..	3	64.8	12.3	Reflux	Water 62%
						5.5	72.5			
7	63.10	3.822	..	..	..	3.3	49.0	12.3	Reflux	
						6	67.0			
8	63.10	3.60	..	..	..	5	64.0	12.3	Reflux	
9	63.10	3.60	..	..	..	24	75.0	12.3	Room	
10	6.15	..	1.35	..	..	5	73.5	8.3	Reflux	
11	6.15	..	1.35	..	..	48	100	8.3	Room	
12	6.15	3.45	..	..	..	4	0	..	Reflux	
13	6.15	3.612	..	..	..	48	0	..	Room	
						168	11.1			
14	63.10	3.822	..	..	..	3	31.0	..	Reflux	93% Alcohol
						16	41.0			
15	6.15	..	1.35	..	..	5	52.0	..	Reflux	
16	6.15	..	1.35	..	..	48	100	..	Room	
17	..	1.911	..	..	60	4.5	3.9	10.2	Reflux	
18	..	1.911	..	..	60	24	14.2	10.2	Room	
19	..	3.822	..	..	220	3	36.7	10.4	..	
						17	41.0			
20	..	..	1.35	..	60	4.5	65.5	10.2	Reflux	Alcohol 38%
21	..	3.822	..	6.15	..	5	0	..	Reflux	Water 62%
22	..	3.822	..	6.15	..	48	0	..	Room	
23	..	3.822	..	12.3	..	5	0	..	Reflux	
24	..	3.822	..	12.3	..	24	0	..	Room	

the initial concentration, and follow the decomposition of both rutin and quercetin. Volumes of 10–20 ml. of the alcoholic solutions of rutin and quercetin were placed in 50-ml. volumetric flasks, and 1–3 ml. of aqueous sodium hydroxide, ethylenediamine or hydrochloric acid was added. The solutions were made to volume with water or SDAG No. 1 G ethyl alcohol. These solutions were refluxed or allowed to stand at room temperature, and the flavonol concentration determined in duplicate in suitably diluted aliquots at various time intervals. When the solutions were refluxed the reaction flasks were immersed in ice water before removing a sample for analysis. The molar composition of the reaction solutions, their initial *p*H values, the temperature conditions, and the percentage decomposition of rutin and quercetin are shown in Table I. It was noted that after an initial loss of about 10%, no further loss of rutin occurred in experiment 2, and that in experiments 6, 7, 8, and 9 the percentage decomposition of rutin varied in each case and was high initially.

Paper Chromatography

The apparatus described by Shephard (3), and Whatman No. 1 paper cut into 3.5 x 28-cm. strips were used in our experiments. The solvent was a mixture of 4 parts *n*-butanol, 5 parts of water, and 1 part of glacial acetic acid as used by Wender and Gage for the separation of flavonoid compounds (4). The procedure and the method for detecting rutin and quercetin on the chromatograms have been previously described by the author (1). No quercetin could be detected on the chromatograms of the alkaline solutions of rutin when samples containing 25–50 μg. of rutin were examined. The chromatograms of similar-sized samples of the acid solutions of rutin showed easily detectable amounts of quercetin.

SUMMARY

1. Rutin has been found to be more stable than quercetin in low concentrations of alkali.

2. The more rapid rate at which quercetin decomposes in alkaline solutions compared to rutin, indicates that the glucoside linkage present in rutin tends to stabilize the flavonol molecule in alkaline conditions.

3. Hydrolysis of rutin occurs readily in acid solutions. Quercetin which is produced by this reaction is not decomposed under these conditions.

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Assay of Cardiotonic Drugs by a Chick Embryo Method*

By GORDON H. BRYAN and CURTIS H. WALDON†

Previous work indicates that chick embryos may be useful as test animals in the bioassay of cardiotonic drugs. By utilizing a three-dose assay design, a technique has been evolved that demonstrates a reasonable correlation of per cent response, dose, and time by chick embryos. A comparison of therapeutic doses of cardiotonic drugs to standard digitalis tincture is closely paralleled by a comparison of chick embryo LD_{50} doses with the same reference standard. In view of these findings, in addition to simplicity of the technique, a chick embryo assay of cardiotonic drugs is proposed.

BECAUSE of their unique position in pharmacology, digitalis and other cardiotonic drugs occupy an impressive portion of biological literature. Many aspects of their cardiac activity have been investigated with particular attention directed toward biological assay. The need for an adequate assay has somewhat diminished since the introduction of relatively pure glycosides, with satisfactory chemical assays. There is as yet, however, no single method for measuring the potency of cardiotonic drugs that appears satisfactory to all concerned. Any assay must satisfy the clinician, the statistician, and the assayist, not to mention the patient. To date, the cat method seems to satisfy the clinician, the frog method finds favor with the statistician, and the present U. S. P. pigeon method is entirely satisfactory to only a few.

It has been said that there is no necessity for the introduction of additional assay methods, but

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† The authors are grateful to the Schering Corporation for a grant to carry on this work and for furnishing olnerin. They also wish to thank the Graduate Research Committee, Montana State University for funds to implement this work.

rather a need for improving the existing ones (1). This would be true were it not for the fact that a major difference exists between the response from cardiotonic drugs injected into the blood stream and that occurring after oral administration with subsequent absorption. This report is based upon the conclusions of DeGraff, Paff, and Lehman (2) concerning the comparison of chick heart response to clinical dosage for various glycosides and the premise that this bioassay should measure the drug absorbed rather than that injected.

Chick embryos have been utilized for investigations of cardiotonic drugs. The most comprehensive papers on chick embryo utility as test material in bioassay have been by Hall (5), Paff (4), and Lehman and Paff (3).

Hall gave an extremely lucid account of the action of the intact seventy-two-hour embryo with and without digitalis. She concluded that an "injected" amount of digitalis could be administered to an embryo, the time for complete stoppage of the heart recorded, the procedure repeated at different dose levels, and a satisfactory assay accomplished.

Lehman and Paff (3) after preliminary work by Paff (4) attempted a two-dose assay correlating the average time for the appearance of an A-V block (atrioventricular block) in excised embryonic hearts and the dose. Again, it was concluded that a satisfactory assay resulted.

The present authors felt that a combination of the technique by Hall (5) and the analysis by Paff (4) would result in a more informative and less difficult assay. Accordingly, in 1948 investigation was started. The technique will be described in detail. It was found that the correlation between the average time for complete stoppage for several hundred hearts on a series of six progressively larger doses of digitalis was not linear. Statistical transformation of this information resulted in an extremely flat slope, with the standard deviation of any of the dose levels, roughly one-half the average time. This indicated a wide variation in response by embryonic hearts to the same dose of digitalis. Since any average time for the appearance of the end point is influenced markedly by the addition of very low or very high recordings, it becomes necessary to use a large number of embryos for each dose level. The results with purified glycosides were somewhat better. It was felt that the average time for the appearance of the end point (complete stoppage) roughly paralleled that of the appearance of an A-V block. However, blocks were difficult to time while observing several embryos, so no attempt was made to correlate the two. In addition to these difficulties, it was found, contrary to good biological procedures, that an upper limit would have to be placed on individual responses included in the data, due to the fact that some embryos were refractory to action of digitalis. In general, the results were similar to those obtained by Paff. This preliminary work seemed to show that there was justification for a bioassay based on a time-dose relationship.

A third factor, that of per cent response, was then considered. The reported experimental results are based upon a graphic-nomographic three-dose assay design by Litchfield and Wilcoxon (6). The present paper may be considered the first of a two-part investigation.

EXPERIMENTAL

The Chick Embryo.—Eggs were purchased from a commercial hatchery. Preliminary work was in agreement with Lehman and Paff (3) that the breed of hens was unimportant. Incubation was carried on at 37° in an ordinary convection-type oven common to most laboratories. Approximately 250 cc. of water in a beaker placed in the oven satisfied humidity requirements. In agreement with Hall (5) it was found that seventy-two-hour embryos or embryos of 40 somites presented a satisfactory test animal.

At this time the heart is well formed, though a double circulation is not yet complete, nor is the valvular control between the four chambers established. Elements of the parasympathetic and sympathetic systems are lacking, which excludes neurogenic complications. Forty-eight-hour embryos are not sufficiently developed to allow adequate macroscopic observation of the heart, and at eighty-four hours the heart has begun its retreat into the thorax and is obscured from view. Preliminary observation indicated that relative to the seventy-two-hour embryo, the eighty-four-hour embryo is more susceptible to the action of cardiotonic drugs, while the forty-eight-hour embryo is more refractory.

Exposure of the Embryo.—About one hour prior to being used, the eggs are placed on end in the incubator with the broad end uppermost. This allows sufficient time for the embryos to migrate to the upper portion of the egg. Exposure of the embryo is readily accomplished by grinding a circular area in the shell around the air pocket with a small electrically driven emery wheel and removing the shell and underlying membrane with tweezers. A frontal view of the clearly defined heart may be seen then. At this stage, any underdeveloped, overdeveloped, or malformed embryos can be discarded. Loss from these causes in addition to nonviability and accident averaged 25%. Eggs from a good commercial source should yield 91% viable embryos. After exposure of the embryos, and in succession, the eggs are placed on a tray constructed to hold them with the long axis perpendicular and submerged to three-fourths of their lengths in the water of a constant temperature bath. The number of eggs accommo-

dated by a bath is dependent on its measurement. A bath 10 in. x 13 in. can easily hold 24 eggs without undue crowding and allowing for variations in size. As stressed by all of the previously mentioned workers, temperature has a very decided influence on cardiac rate and subsequent absorption of drugs. High temperatures increase the rate, while low ones decrease or stop the beat. In agreement with Lehman and Paff (3), 37° was chosen for the temperature of the water bath primarily because it was more "physiologic."

The hearts of embryos so treated will continue beating regularly but more slowly than normal for four to twenty-four hours.

Administration of Drugs.—Hall (5) placed an upper limit of 14% on the concentration of alcohol that can be brought into contact with the embryo without appreciably altering cardiac activity or survival time. It was determined that a 10% alcoholic solution was satisfactory, so all the cardiotonic drugs used were in that concentration. The glycosides were dissolved in 95% alcohol and adjusted by dilution with normal saline. Strophanthus and digitalis as tinctures were evaporated and restored to the desired volume with normal saline. The cardiotonic drugs in solution were administered by means of a 1-cc. pipette calibrated in hundredths. The dilution of cardiotonic drug was allowed to flow over the surface of the embryo and remained in the pocket formed by the shell, egg yolk and white, and the embryo. The pocket can accommodate a maximum of 0.5 cc. of fluid. However, 0.2 cc. appeared to be the most satisfactory from the standpoint of volume and ease of pipetting, consequently a constant volume of 0.2 cc. was adopted for all doses. Delivery of a particular dose to 10 or more embryos can be rapidly accomplished.

Timing.—Timing, by means of an electric timer calibrated in seconds, was started from the onset of pipetting. The slight time lag between the first and last embryo to receive a given dose of a drug was disregarded. Responses were determined at measured intervals.

End Point.—As mentioned previously, there did seem to be some correlation between the appearance of an A-V block and complete stoppage of the heart. However, any attempt to measure it with reasonable accuracy while watching several embryos was too difficult, so that complete stoppage was chosen as the end point. This eliminated any discrepancies due to individual interpretation in addition to being a quantal response. Observation is macroscopic and certain, since a heart has either stopped beating or it has not.

OBSERVATIONS

The proposed technique involves three variables: the dose range, the time elapsing before reading the response, and response to three different dose levels falling between 16 and 84%. The results for U. S. P. reference standard digitalis as a tincture are summarized in Table I. The times listed are dictated by the response.

The reported work was done in November and December to preclude seasonal variations as suggested by Lehman and Paff (3). Our preliminary observations did not substantiate their opinion that a variation existed between (summer, winter, and fall) embryos.

TABLE I[a]

Time, Sec.	Animals/ Dose	Ref. Std. Dig., Cc.	S	f_s	LD_{50}, Cc.	$f_{LD_{50}}$
900	10	0.40 0.50 0.60	1.25	1.20	0.35	1.18
600	10	0.40 0.50 0.60	1.29	1.16	0.44	1.12
450	10	0.40 0.50 0.60	1.29	1.11	0.52	1.10

[a] Chi^2 fit satisfactory for all curves.

TABLE II[a]

Drug	Animals/ Dose	Time, Sec.	Dose, Mg.	S	f_s	ED_{50}, Mg.	$f_{ED_{50}}$
Digitoxin	10	450	0.04 0.05 0.06	1.45	1.37	0.055	1.14
Digoxin	10	450	0.40 0.60 0.80	1.56	1.51	0.490	1.46
Olnerin	10	450	0.04 0.05 0.06	1.38	1.25	0.046	1.12
Strophanthus[b] tincture	10	450	0.20 0.30 0.40	2.3	5.1	0.190	1.70

[a] Chi^2 fit satisfactory for all curves.
[b] Dose and LD_{50} in cc. of tincture.

TABLE III

Drug	N. F. and U. S. P. Therapeutic Dose, Mg.	Ratio[a] to Dig. Tr.	Chick Embryo LD_{50}, Mg.	Ratio[b] to Dig. Tr.	19/20 Confidence Limits
Digitalis Tr.	100.0	...	52.0	...	...
Digitoxin	0.1	1000.0	0.042	1238.0	1529-981
Digoxin	0.5	200.0	0.490	106.0	155-72
Olnerin	0.1[c]	1000.0	0.046	1130.0	1253-1019
Strophanthus Tr.	50.0	2.0	19.0	2.7	4-1

[a] Dig. Tr. therap. dose (mg.)/Drug therap. dose (mg.).
[b] Digitalis Tr. LD_{50} to chick embryo (mg.)/Drug LD_{50} to chick embryo (mg.).
[c] Assumed.

All three time readings for the same dose range approximate a *b* value of 12, however, the 19/20 confidence limits for the ED_{50} appear to be narrowed for a time interval of four hundred and fifty seconds. Due to limited circumstances, the day to day or week to week changes that might occur in responses to the same doses were not investigated. In view of the information presented, and with a potential method of bioassay in mind, four hundred and fifty seconds was chosen as the standard time for the response to be determined. A few preliminary observations established the dose ranges yielding adequate responses for strophanthus tincture, digitoxin, digoxin, and olnerin. The results are summarized in Table II. There was no attempt made to investigate changes occurring at increasing dose ranges for each of these drugs. It is probable that the values for the slopes could be increased with increasing doses, but it would be necessary to adjust to a shorter time. However, the distribution of responses seems more desirable at the dose ranges shown.

The 19/20 confidence limits for the ED_{50} of digitoxin (±14%) and olnerin (±12%) parallel the limits for digitalis tincture. Those for digoxin (±40%) and strophanthus tincture (±70%) are extremely high. The reason for this is not known; however, there should be additional investigation as to the effect of varying dose ranges and time intervals on these two drugs.

The range for strophanthus tincture is at a maximum, since any increase results in 90 to 100% response for all three doses within the specified time limit. Both strophanthus and digoxin seem to be troublesome in most bioassays.

Table III is a comparison of U. S. P. average therapeutic doses using digitalis tincture as a point of reference, and the relative potencies found by the chick embryo method. It may be seen that there is good agreement for all drugs, with the single exception of digoxin. This exception was not anticipated in view of the findings of DeGraff, Paff, and Lehman (2). Their comparison was with a different point of reference and was based on digitalizing dosage rather than an average dose.

CONCLUSIONS

A technique has been described for the assay of cardiotonic drugs using intact chick embryonic hearts. A three-dose pattern of assay has been utilized. A good correlation has been established between the therapeutic doses and the chick embryo LD_{50} of the drugs tested. It appears that the intact chick embryonic heart affords a simple, cheap, and reliable medium for the evaluation of cardiotonic drugs.

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American Association for the Advancement of Science—Pharmacy Subsection Meeting

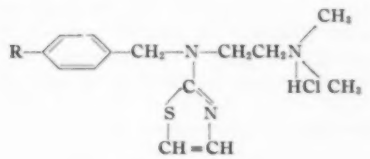
The next meeting of the American Association for the Advancement of Science will be held in Philadelphia on December 26–31, 1951. The program of the Pharmacy Subsection of the Medical Sciences is being made up. Six sessions of this Subsection will be held. Members are invited to attend the meetings of the Section on Medical Sciences, Subsection on Medicine, and the Section on Chemistry.

Microtoxicology. IX. The Effect of *para* Substitution in Three New Thiazole Antihistaminics upon Their Reactivity to Microchemical Tests*

By THOMAS J. HALEY† and GEO. L. KEENAN‡,§

It has been shown that *para* substitution in the benzyl nucleus of a new series of antihistaminics definitely changes the reactivity of the compounds to colorimetric microtoxicological reagents. Such substitution has little or no effect on the reactivity of these antihistaminics to the usual precipitation reagents. The tests reported enable the compounds to be identified and differentiated from one another by a simple procedure. However, the optical crystallographic properties of these antihistaminics offer the best means for identifying and differentiating them, not only from each other, but from other organic compounds.

AN EXAMINATION of our past microchemical work on the identification of the various antihistaminic agents reveals that slight changes in substituent groups on the same molecule result in detectable changes in chemical reactivity (1–5). However, this past work did not include sufficient compounds in which the changes were in a single position; thus it was not possible to conclude that any of the differences in chemical reactivity seen were due to substitution in a particular position. Recently we have been able to obtain a new series of antihistaminics in which the basic molecule has been varied in only one position. Their general formula is given below and substitution is in the *para* position of the benzyl nucleus.



Drug	R
194-A	H
194-B	CH_3O
194-C	Cl

Upon the basis that single position substitution would change the chemical reactivity of the compounds, we have investigated their reactivity to the usual microtoxicological colorimetric and precipitation reagents.

EXPERIMENTAL

The uncorrected melting points of the drugs were determined with a Melt-Pointer electrically heated capillary tube melting point apparatus employing Anschutz thermometers: 194-A, 171 to 172.5°; 194-B, 162 to 164°; 194-C, 149 to 151°.

The reagents employed were prepared as described by Thienes and Haley (6), Autenrieth and Bauer (7), and Rosenthaler (8). The compounds were tested by placing one drop of reagent on a microscope slide or in the well of a spot-plate and adding about 0.1 mg. of the drug to it. At times, when using the precipitation agents, the above amount of drug was dissolved in one drop of distilled water and then mixed with the reagent by streaking. This procedure, which results in three zones, enables a better comparison to be made between the pure crystals of the reactants and the resultant precipitate when the slides are dried and set aside for future study. All observations were for a period of thirty minutes and, in the case of the acid colorimetric reagents, the end point was the formation of a stable color or a charring of the drug. However, only the colors appearing prior to charring were used for identification purposes.

As a further means of identification, photomicrographs of the pure antihistaminics were taken with an Erb and Gray Visicam photographic attachment on a Band L monocular microscope using the 16-mm. objective and the 10× eyepiece. All the drugs were crystallized from anhydrous ether.

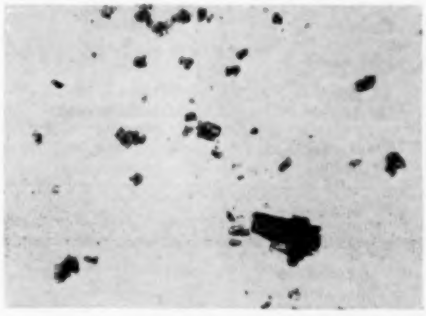


Fig. 1.—Photomicrograph of 194-A.

* Received February 26, 1951, from the School of Medicine, University of California at Los Angeles.

† School of Medicine, University of California at Los Angeles.

‡ Formerly Microanalyst, United States Food and Drug Administration. Deceased.

§ The authors wish to thank White Laboratories, Inc., for the antihistaminics used in this study.

Fig. 2.—Photomicrograph of 194-B.

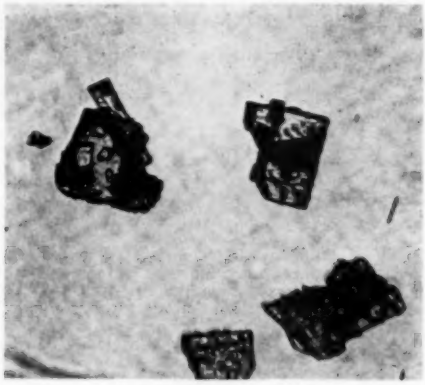


Fig. 3.—Photomicrograph of 194-C.

RESULTS

The results obtained with the colorimetric reagents are given in Table I. Granular amorphous precipitates were obtained with the following precipitation reagents: antimony trichloride 1%, chloroauric acid 5%, saturated aqueous solution of picric acid, Dragendorff's, Marme's, Wagner's, Sonneschein's, Scheibler's, Mayer's, sodium nitroprusside 1%, and antimony potassium iodide. Chloroplatinic acid 5% gave thorn-apple bundles of needles with 194-A; bundles of coarse feather-like crystals in sheaves with 194-B; and an amorphous precipitate with 194-C.

The photomicrographs of the pure antihistaminics indicated that *para* substitution had changed the crystalline characteristics of the compounds to some extent because 194-A and 194-B consisted of rectangular plates, some of which were elongated (apparently belonging to the orthorhombic system), while 194-C consisted of quadratic plates with several broad elongated forms. These characteristics in themselves are not conclusive but are additional information useful, in combination with the microchemical data, for identifying and differentiating these compounds from one another.

DISCUSSION

From the results in Table I, it appears that substitution in the *para* position of the benzyl nucleus with chlorine blocks the reactivity of the drug (194-C) to most of the colorimetric reagents used. However, this is advantageous because, although the drug reacts differently only with Mandelin's reagent, this test when combined

TABLE I.—COLORIMETRIC REACTIONS OF THIAZOLE ANTIHISTAMINICS

Reagent	194-A HCl	194-B HCl	194-C HCl
Concd. HNO_3	Pale yellow changing to orange	Magenta changing to carmine	Colorless
Concd. H_2SO_4	Pink changing to pale yellow	Pale yellow	Pale yellow
Buckingham's	Colorless	Magenta	Colorless
Frohde's	Colorless	Fuschia changing to magenta	Colorless
Mandelin's	Dirty brown	Brilliant carmine with a purple center	Light orange changing to red
Marquis'	Orange brown	Magenta	Colorless
Mecke's	Salmon pink	Magenta	Colorless
	Brown	Magenta	Brown
Sonneschein's ceric reagent	No change	Purple	No change

TABLE II.—OPTICAL CRYSTALLOGRAPHIC PROPERTIES OF THE THIAZOLE ANTIHISTAMINICS

Compound	Habit	Optical Sign	2E	Refractive Indices		
				α	β	γ
194-A HCl	Plates	(−)	90 ± 5°	1.602	1.631	1.640
194-B HCl	Plates	No figures	....	1.602	..	1.640
194-C HCl	Plates	(+)	15-20°	1.602	1.608	1.660

with the others allows the drug to be differentiated from the others of the 194 series.

Methoxyl substitution in the same position, while it does not block chemical reactivity, changes it sufficiently so that 194-B is readily identified and differentiated from both 194-A and 194-C.

The unsubstituted compound 194-A is extremely reactive to the microchemical reagents used so that no difficulties should be encountered in identifying and differentiating this compound from others of the series. It is evident that, for the most part, the precipitation reagents are of little value in determining *para* substitution because these antihistaminics were in general as unreactive as the other compounds previously investigated (1–5). However, chloroplatinic acid did form different crystalline precipitates with 194-A and 194-B, and these precipitates could be used as a further means for identification and differentiation of these compounds.

The colorimetric and precipitation reactions of the thiazole antihistaminics are useful in differentiating these compounds from one another but they are inconclusive when comparisons are made with the reactions given by other antihistaminics and alkaloids. In fact, the reactions are very similar to those previously reported (1–5). However, it is possible to definitely identify and differentiate the thiazole antihistaminics, not only from each other, but from other antihistaminics and alkaloids by use of their optical crystallographic properties. Table II from Keenan and Haley (9) gives the optical crystallographic properties as determined by the immersion method.

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Acute and Short-Term Toxicity Studies of Fused Bentonite Sulfur Preparations*

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The results of the determination of the acute toxicity of fused bentonite sulfur emulsion (FBSE), alone and in combination with Quadrafos and a liquid concentrated base (LC) are reported. The animals employed include rats, mice, and rabbits. All of the preparations studied exhibited a low order of toxicity.

THE USE of fused bentonite sulfur as a therapeutic agent in the treatment of acne vulgaris, seborrheic dermatitis, and dermatophytosis (1) has focused attention on this fungicidal agent (2). As Sternberg and Taylor point out, the aqueous base has been found by Dowdy and the Vincents (3) to have unusual properties. The toxicity studies reported herewith establish the fused bentonite sulfur preparations and the aqueous base as materials of low toxicity.

MATERIALS

Three preparations were studied: (*a*) fused bentonite sulfur emulsion (FBSE); (*b*) fused bentonite sulfur emulsion with Quadrafos[1] (FBSQ); and (*c*) a liquid concentrated base (LC). Bentonite (Wilkinite type) was heated with sulfur[2] and fused by the Niagara Sprayer Company; the emulsion contains 12% in the fluid base. The concentrated fluid base

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[1] Quadrafos is the trade mark name of Rumford Chemical Works, Rumford, R. I., for sodium tetraphosphate ($Na_6P_4O_{13}$).

[2] Toxicity after ingestion of sulfur by man is largely unknown. Toxic symptoms, followed by an uneventful recovery, were reported in one man who ingested 60 Gm. in a twenty-four-hour period. Men have ingested 500 to 750 mg. of colloidal sulfur daily for ten days without unpleasant subjective reactions. Poisonings have been reported from the use of sulfur ointment on children with extensive scabies (16–26).

contains quince seed extract, urea,[3] ethanol,[4] bile salts,[5] and water; dilution with an equal volume of water makes the regular fluid base.

ACUTE TOXICITY TESTS

Estimations of the acute toxicity were made for each of the three materials administered intraperitoneally to rats, mice, and rabbits and given orally in a single dose (by stomach tube) to rats. The rats were adult albino animals mostly females; no sex differences in toxicity were observed so that the data have been combined. The mice were adult female albinos. The rabbits were adult male albinos, black, brown, and varicolored. Mortality records were kept daily for a period of at least three weeks after treatment. The data are summarized in Tables II–V. The LD_{50}'s have been estimated graphically and are presented in Table I.

TABLE I

Material	Intraperitoneal LD_{50} Rats, Cc./Kg.	Mice, Cc./Kg.	Rabbits, Cc./Kg.	Oral LD_{50} Rats, Cc./Kg.
FBSE	4.5	5	0.2–0.5	50[a]
FBSQ	2.5	2	0.2–0.5	40[a]
LC	6.0	5.5	5–7.5	40

[a] No deaths.

FBSE may be described as practically nontoxic when given intraperitoneally to rats and mice, but it is moderately toxic in the more susceptible rabbits. The toxicity of FBSQ is somewhat greater than that of FBSE; when given intraperitoneally to rats and mice, it may be classed as slightly toxic. In rabbits, the toxicity of FBSQ is moderate. Following intraperitoneal administration, LC is practically nontoxic in rats, mice, and rabbits. All three substances, when given orally to rats, may be administered without mortality in such large doses that they may all be classed as relatively harmless.

Following the intraperitoneal administration of FBSE, some rats were observed for as long as one hundred and four days. On autopsy, grayish material streaked with yellow was frequently found in cyst-like membranes attached to the upper abdominal wall. Some adhesions were also observed tying the gut and other visceral organs together. The same general findings were observed on autopsy of the animals given FBSQ. The cheesy material was absent in the peritoneal cavities of rats that had been given LC; however, adhesions were sometimes seen. Similar abnormalities were observed in mice and in rabbits. There were no evidences of gross abnormalities due to treatment in the rats given oral doses of either of the three materials.

The LD_{50}'s are large in each instance. The oral toxic doses are very large. The differences between

[3] Urea is toxic only in extraordinarily large doses; toxic symptoms in man occur with blood levels above 165 mg. % (4–6).

[4] No attempt will be made to review the extensive literature on the toxicity of ethanol.

[5] The toxicity of the bile salts roughly parallels the degree of reduction and, conversely, decreases with oxidation. The parenteral toxicity is much greater than the oral, depending on the nature of the bile acid. The average lethal dose, parenterally, varies from the order of 10 mg./Kg. to more than 1 Gm./Kg. (7–15).

TABLE II.—MORTALITY FOLLOWING INTRAPERITONEAL INJECTION IN RATS OF FBSE, FBSQ, AND LC

Material	Dose, Cc.	No. Rats	Av. Body Wt., Gm.	Dose, Cc./Kg.	Mortality, %
FBSE	0.2	12	184	1.1	25
	0.4	12	187	2.2	83
	0.8	12	194	4.2	16
	1.0	10	200	5.2	80
	1.2	7	190	6.5	100
	2.0	12	196	10.4	83
FBSQ	0.1	2	222	0.4	0
	0.2	10	262	0.8	0
	0.4	15	230	1.7	50
	0.5	2	254	2.0	0
	0.6	10	205	3.0	60
	0.8	15	220	3.7	70
	1.0	12	204	5.0	67
	1.2	15	242	5.1	80
	2.0	15	244	8.4	90
	3.0	5	207	1.46	100
LC	0.1	2	237	0.4	0
	0.5	2	206	2.4	0
	1.0	22	249	4.1	5
	1.2	15	262	4.6	27
	1.4	15	265	5.3	27
	1.6	15	271	5.9	40
	1.8	15	215	6.4	100
	2.0–10.0	15	211	10–19	100

TABLE III.—MORTALITY FOLLOWING INTRAPERITONEAL INJECTION IN MICE OF FBSE, FBSQ, AND LC

Material	Dose, Cc.	No. Mice	Av. Body Wt., Gm.	Dose, Cc./Kg.	Mortality, %
FBSE	0.01	2	22	0.5	0
	0.02	17	28	0.7	0
	0.04	17	26	1.6	6
	0.08	17	25	3.2	18
	0.12	16	25	5.0	94
	0.2–1.0	6	18	11–62	100
FBSQ	0.01	2	24	0.4	0
	0.02	17	28	0.7	0
	0.04	17	29	1.4	6
	0.08	17	27	3.0	82
	0.12	16	25	5.0	100
	0.2–0.5	4	24	9–21	100
LC	0.01	2	23	0.4	0
	0.05	7	23	2.2	0
	0.06	10	22	2.7	0
	0.10	15	23	4.4	20
	0.12	10	24	5.0	67
	0.14	10	23	6.9	60
	0.15	15	22	7.0	73
	0.20	17	22	9.2	95
	0.5–1.0	4	23	23–42	100

TABLE IV.—MORTALITY FOLLOWING INTRAPERITONEAL INJECTION IN RABBITS OF FBSE, FBSQ, AND LC

Material	Dose, Cc.	No. Rabbits	Av. Body Wt., Kg.	Dose, Cc./Kg.	Mortality, %
FBSE	0.3–1.2	2	4.1	0.1–0.2	0
	1.9–45.0	6	4.5	0.5–10.0	84
FBSQ	0.5–1.2	2	4.8	0.1–0.2	0
	2.6–22.0	4	4.8	0.5–5.0	100
LC	12–21	2	4.6	2.5–5.0	0
	38–42	2	4.6	7.5–10	100

TABLE V.—MORTALITY FOLLOWING SINGLE ORAL DOSES IN RATS OF FBSE, FBSQ, AND LC

Material	Dose, Cc.	No. Rats	Av. Body Wt., Gm.	Dose, Cc./Kg.	Mortality, %
FBSE	2.0	10	162	12.4	0
	6.0	15	158	38.0	0
	8.0	15	150	50.0	0
FBSQ	0.1	2	177	0.6	0
	0.5	2	221	2.3	0
	1.0	2	183	5.5	0
	2.0	2	216	9.2	0
	3.0	2	233	13.0	0
	5.0	16	174	29.0	0
	8.0	15	182	44.0	0
LC	0.1	2	193	0.5	0
	0.5	2	219	2.3	0
	1.0	2	201	5.0	0
	2.0	2	225	8.9	0
	3.0	2	197	15.0	0
	4.0	2	190	21.0	0
	5.0	17	209	24.0	0
	6.0	10	195	31.0	0
	8.0	15	176	46.0	67

the three materials are not great and, except for the susceptibility of the rabbit to FBSE and FBSQ, the species variations are not important. In general, the acute toxicities of these three materials are low.

SHORT-TERM STUDIES IN RATS

Groups of male and female rats were fed diets containing various levels of FBSE, FBSQ, and LC for periods of sixty days and observed for various criteria of toxic effects. In general, large doses could be ingested daily with very little evidence of toxic effects (Table VI and VII).

Growth curves showed that the rats (10 males and 10 females per group) given fox chow meal with meat into which 1% and 10%, respectively, of FBSE, FBSQ, and LC were mixed grew as well as the control groups. When diets containing 30% were given, some growth retardation was observed in each case. The average initial and final body weights are given in Tables VI and VII. In Fig. 1 the average growth curves for the male rats have been plotted; the female rats exhibited comparable patterns. The fact that such a high percentage of these three materials was tolerated with only moderate diminution in growth should be emphasized. Few materials can be ingested by rats at a level of 30% of the diet without producing violent and intractable diarrhea.

Mortality was negligible; only two of the 240 rats died during the sixty-day period.

Near the end of the experimental period, a single pooled urine sample was collected from each group by placing several rats in a single metabolism cage overnight. The customary semiquantitative analyses for sugar and protein gave only values such as are usually found in control rats. There was no evidence of any effect on the urinary concentrations of these constituents with increase in percentage of any of the three materials.

Blood samples were taken near the end of the period for hematological observations. The hemoglobin values, red blood cell counts, white blood cell counts and differential counts were comparable

TABLE VI.—SUMMARY OF OBSERVATIONS ON MALE RATS GIVEN VARIOUS DIETS AS INDICATED FOR 60 DAYS

Diet	Av. Body Wt., Gm.		Mortality	Urine Analysis	Hematology	Pathology				
	Init.	Final				Av. Liver Wt.	Related to Treatment	Lung Infection	Renal Infection	Other
Control	83	242	0/10	Normal	Normal	9.54	None	1	1	...
1% FBSE	87	246	0/10	Normal	Normal	10.13	None	1	0	Testicular atrophy
10% FBSE	80	243	0/10	Normal	Normal	11.83	None	0	0	...
30% FBSE	87	175	0/10	Normal	Normal	10.12	None	0	0	...
Control	81	246	0/10	Normal	Normal	10.15	None	2	0	...
1% FBSQ	80	240	0/10	Normal	Normal	9.09	None	1	0	...
10% FBSQ	76	242	0/10	Normal	Normal	9.62	None	0	0	...
30% FBSQ	81	190	0/10	Normal	Normal	11.16	None	0	0	...
Control	68	256	0/10	Normal	Normal	9.67	None	3	1	...
1% LC	66	232	0/10	Normal	Normal	9.44	None	0	0	...
10% LC	63	230	0/10	Normal	Normal	9.64	None	0	0	...
30% LC	60	183	0/10	Normal	Normal	8.61	None	4	0	...

TABLE VII.—SUMMARY OF OBSERVATIONS ON FEMALE RATS GIVEN VARIOUS DIETS AS INDICATED FOR 60 DAYS

Diet	Av. Body Wt., Gm. Init.	Av. Body Wt., Gm. Final	Mortality	Urine Analysis	Hematology	Av. Liver Wt.	Pathology Related to Treatment	Pathology Lung Infection	Pathology Renal Infection	Pathology Other
Control	73	175	0/10	Normal	Normal	6.54	None	0	0	...
1% FBSE	74	177	0/10	Normal	Normal	7.80	None	1	0	...
10% FBSE	76	179	0/10	Normal	Normal	7.05	None	0	0	...
30% FBSE	70	152	0/10	Normal	Normal	7.54	None	0	0	...
Control	76	198	0/10	Normal	Normal	7.84	None	2	0	...
1% FBSQ	76	180	0/10	Normal	Normal	6.84	None	0	0	1 Renal parasite
10% FBSQ	74	183	0/10	Normal	Normal	7.54	None	1	0	...
30% FBSQ	74	155	0/10	Normal	Normal	8.74	None	0	0	1 Liver focal necrosis
Control	66	210	0/10	Normal	Normal	8.40	None	4	0	...
1% LC	60	181	0/10	Normal	Normal	7.13	None	0	0	...
10% LC	62	170	0/10	Normal	Normal	6.84	None	2	0	...
30% LC	65	174	0/10	Normal	Normal	7.24	None	2	0	...

TABLE VIII.—SUMMARY OF OBSERVATIONS ON DOGS GIVEN VARIOUS DOSES OF MATERIALS AS INDICATED FOR 60 DAYS

Materials	Dose, Gm./Kg./Day	Av. Body Wt., Kg. Init.	Av. Body Wt., Kg. Final	Urine Analysis	Hematology	Liver Weight, Gm./Kg.	Histopathological Observations
FBSE	5	20.2	17.2	Normal	? Decrease RBC + Hgb.	22.2	Slight testicular atrophy
	2	18.4	18.7	Normal	Normal	24.7	Intestinal parasites; chronic kidney inflammation in submucosa of pelvis
FBSQ	0.5	9.4	9.7	Normal	Normal	20.6	Normal
	5	9.7	12.0	Normal	Normal	23.0	Normal
	0.5	9.0	8.2	Normal	? Decrease RBC + Hgb.	23.2	Normal
LC	5	17.0	19.1	Normal	Increase RBC + Hgb.	18.3	Normal
	0.5	8.8	8.9	Normal	Normal	24.3	Normal

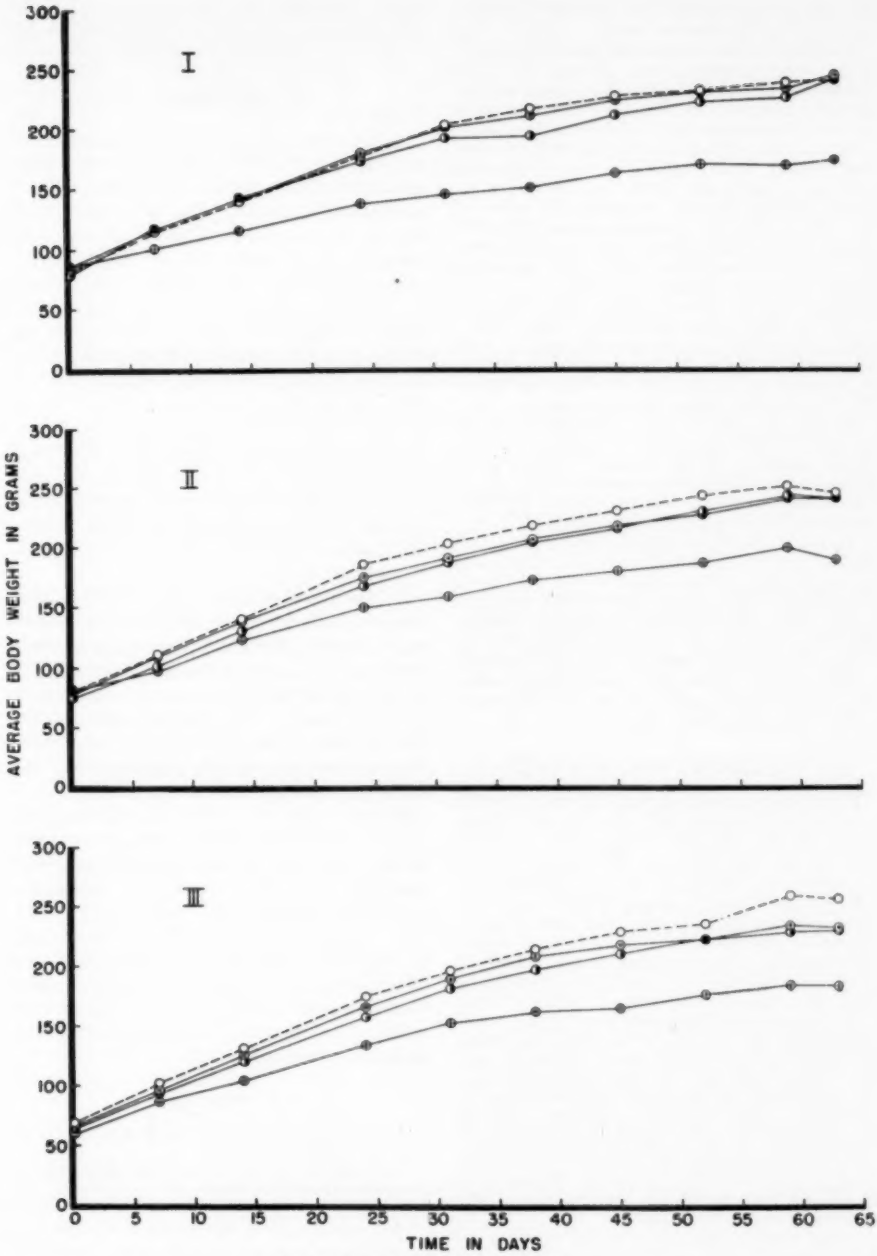

Fig. 1.—Growth curves for male rats.

I, Fused bentonite sulfur emulsion; II, bentonite sulfur with Quadrafos; III, concentrated base. ○ = controls, ⊙ = 1%, ◑ = 10%, ⊕ = 30%.

from group to group and within the normal limits of such observations in our rat colony.

At autopsy, organ weights were recorded for heart, lungs, spleen, stomach, liver, kidney, brain, and testes. Average organ weights were compared, both directly and when expressed as mg./Gm. body weight. There was evidence of dependence of organ weight on treatment only in the case of the average liver weights; these tended to increase in both male and female rats given FBSE and FBSQ (for the latter especially at 30%), whereas in rats given LC the liver weights may be slightly smaller in the 30% groups. The average liver weights for each group are given in the summary tables. The significance of the liver weight changes is unknown.

Tissue samples of the following organs were prepared for histological examination: heart, lungs, liver, spleen, kidneys, reproductive organs, bone marrow, and brain. The histological examinations were made by Dr. David R. Hawkins and Edwin H. Church of the Department of Pathology. They concluded that FBSE, FBSQ, and LC as administered produced no specific histological changes. In the summary tables the most frequently described minor pathological abnormalities are listed; a number of lung infections were seen, a few renal infections, and some parasitic infestations.

To summarize the feeding studies in rats, it may be said that FBSE, FBSQ, and LC exhibit a low order of toxicity. The addition of even 10% in the diet did not depress the growth rate and 30% produced only moderate growth retardation. Mortality was negligible. Only normal values were recorded from urine analyses and hematological studies. Organ weights were mostly normal; some variation in liver weights was found. No specific pathological changes were observed.

SHORT-TERM FEEDING STUDIES: DOGS

Dogs were given various doses of FBSE, FBSQ, and LC for a period of sixty days with the findings summarized in Table VIII. Doses of 5 and 0.5 Gm./Kg./day of each material were given to one dog and, in addition, one dog was given 2 Gm. of FBSE/Kg./day. In most cases the dogs gained a little weight and were in good condition with good appetite throughout the experimental period. Urine samples were analyzed at the beginning of the experimental period and near the end; only normal values for sugar and protein were obtained. Two or three blood samples were taken from each dog for hematological study; one sample was taken just before beginning the test feeding, the second sample after six to eight weeks, and the third sample just prior to termination. In three dogs, minor variations in red blood cell counts and hemoglobin were recorded, in the other dogs only normal values were found. At the time of autopsy, organ weights were recorded. These were all found to be normal; however, for comparison with the rat liver weights, the dog liver weights are recorded in Table VIII.

Tissue samples were taken of the following organs: heart, lungs, spleen, stomach, small and large intestine, pancreas, liver, adrenal, kidney, reproductive organs, bone marrow, and brain. The histological examinations were conducted by Drs. D. H. Hawkins, Harvey J. Blanchet, Jr., and Edwin H. Church of the Department of Pathology. They found no evidence of pathological changes attributable to the administration of FBSE, FBSQ, or LC. A few minor pathological changes were noted.

SUMMARY

1. The acute toxicity of fused bentonite sulfur emulsion (FBSE), fused bentonite sulfur emulsion with Quadrafos (FBSQ), and a liquid concentrated base (LC) has been measured in rats, mice, and rabbits. The LD_{50}'s are of the following magnitudes: (*a*) in rats, intraperitoneal administration: FBSE, 4.5 cc./Kg.; FBSQ, 2.5 cc./Kg.; LC, 6.0 cc./Kg.; given orally, FBSE, 50 cc./Kg. caused no deaths; FBSQ, 40 cc./Kg. caused no deaths; LC, 40 cc./Kg.; (*b*) in mice, intraperitoneal administration: FBSE, 5 cc./Kg., FBSQ, 3 cc./Kg.; LC, 6 cc./Kg.; (*c*) in rabbits, the lethal ranges following intraperitoneal administration: FBSE and FBSQ, 0.2 to 0.5 cc./Kg., respectively; LC, 5 to 7.5 cc./Kg. FBSE is practically nontoxic to rats and mice but is moderately toxic to rabbits. FBSQ is somewhat more toxic than FBSE. LC is practically nontoxic.

2. Groups of rats, 10 males and 10 females, were maintained for sixty days on diets containing 0 (control groups), 1, 10, and 30 per cent, respectively, of each of the three preparations. Some growth retardation was observed in the 30 per cent groups; the other experimental rats grew as well as the control groups. No effects were observed on survival, urine sugar and protein, or on hematological values.

Organ weights were within normal ranges except for a tendency for liver weights to vary with dosage. No specific histological changes were produced. The three preparations exhibited a low order of toxicity.

3. Dogs were fed doses of from 0.5 to 5 Gm./Kg./day of each preparation for sixty days. No effects were observed on body weights, urine sugar and protein, hematological values, or organ weights. No pathological changes were attributed to the treatment.

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A New Hydrocarbon Ointment Base: Jelene 50W*

By E. R. JONES† and B. LEWICKI

A new ointment base known as Jelene 50W and consisting essentially of saturated hydrocarbons has been used in place of petrolatum as an ingredient of the bases for several official and unofficial ointments. Ointments thus prepared have been compared over a period of several months with the same ointments containing petrolatum. The conclusion reached as a result of this study is that Jelene is preferable to petrolatum because of its high stability over a wide temperature range.

JELENE 50W[1] is a white, unctuous substance having a soft ointment consistency and a Wiley melting point of 90–91° C. It is described by the manufacturers as "consisting essentially of saturated hydrocarbons." The consistency of Jelene 50W remains nearly unchanged within the temperature range of −40° F. and 180° F. and it was this unusual property that suggested the new substance as a base for official ointments.

We had been requested to cooperate in a field testing program to develop a suitable base for veterinary ointments capable of retaining their desired consistency over a wide temperature range. Fortunately, this program offered an opportunity to test certain more common ointments in which the white petrolatum had been replaced with Jelene 50W. However, we shall limit this report to the behavior of six U. S. P. XIII and seven N. F. VIII ointments of wide variety as they were prepared and tested in our Product Testing Laboratory.

EXPERIMENTAL

The 13 official ointments were chosen for this test because they were representative of widely different medicaments and because they were composed of high proportions of various bases which we wished to replace with Jelene 50W. The various official ointments were prepared as controls.

Each of the Jelene 50W ointments, with controls, was placed in 1-oz. flint glass jars to be stored four months at various temperatures. Routine periodic inspections were made of the preparations as they were held at 40° F., room temperature (approx. 75° F.), 100° F., 110° F., and 120° F.

A study of these tabulations shows that except in a very few instances, an ointment was obtained with Jelene 50W which we thought was superior in appearance, ease of application, and stability at elevated temperatures to the official ointments.

Their ability to maintain their approximate original consistency at both very low and very high temperatures and to easily issue from a tube with a very small opening is an asset we can all appreciate, and should be of value particularly in opthalmic ointments.

The ease with which it can be blended with other ingredients on the ointment slab will make it a popular product for prescription work.

In the present form, Jelene 50W has the limitation of dissolving such substances as menthol, methyl salicylate, and camphor with the result that ointments containing these chemicals become too soft. Likewise, coal tar produces an ointment too soft to prevent separation. Jelene 50W does not lend itself to the incorporation of waxes as stiffening agents because it is difficult to cool the resulting mixture to a smooth consistency. The manufacturers advise us, however, that this base can be prepared of increased stiffness, if desired.

It was also noted that excessive milling decreased the consistency of this base. In an effort to evaluate this change, we passed samples of Jelene 50W six times through an ointment mill and a roller mill. Penetrometric measurements on these samples, when compared with unmanipulated Jelene

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[1] Supplied by the Research Products Corporation, Madison, Wis.

TABLE I.—U. S. P. XIII OINTMENTS

Name	Official Base	Appearance of Official Prepn.	Appearance of Jelene Prepn.	Breaking Temp. Official Oint., ° F.	Breaking Temp. Jelene Oint., ° F.
Ammoniated mercury ointment	White ointment and wool fat	Ivory	Soft, white, glossy	110	120
Zinc oxide ointment	White ointment and wool fat	White, flat	White, glossy	110	120
Sulfur ointment	White ointment and wool fat	Yellow	Soft, lighter yellow, glossy	110	120
Belladonna ointment	Yellow ointment	Soft, greenish black	Soft, greenish black	110	120
Phenol ointment	White ointment	Light yellow	Softer, white and translucent	120	120
Coal tar ointment	White petrolatum	Gray, stiff	Soft gray which later separates	110	110 (more pronounced)

TABLE II.—N. F. VIII OINTMENTS

Name	Official Base	Appearance of Official Base	Appearance of Jelene Base	Breaking Temp. Official Base, ° F.	Breaking Temp. Jelene Base, ° F.
Ointment benzoic and salicylic acids	White petrolatum and wool fat	Ivory	Soft, white, glossy	110	120
Ichthammol ointment	Petrolatum and wool fat	Black	Rich, soft, black	120 (slight)	120
Calamine ointment	Petrolatum, wool fat, and yellow wax	Stiff, pink	Soft, pink (very slightly darker)	110 (marked)	120 (slight)
Iodine ointment	Yellow ointment	Soft, black	Soft, black with slightly greater violet hue	120 (pockets)	120
Compound resorcinol ointment	Petrolatum, wool fat, and yellow wax	Stiff, gray. Discolors	Slightly darker, softer, more attractive. Discolors	100 (progresses at higher temperatures)	75 (slight pockets form but no increase at higher temperatures)
Capsicum ointment	Petrolatum and paraffin	Orange color, semitransparent	Soft, transparent red-amber (superior to official)	120	120
Mild calomel ointment	White petrolat. hyd. wool fat and water	Ivory	Soft, white	110 (marked at 120)	120[a]

[a] Prepared with hydrophilic Jelene w/o type.

50W, showed the viscosity of the sample passed through the roller mill to be essentially unchanged but the viscosity of the sample passed through the ointment mill had been considerably decreased.

It probably will never be possible to reduce the cost of Jelene 50W to compare with the cost of white petrolatum. However, we feel that the physical advantages offered by this new ointment are sufficient to suggest that it be substituted for white petrolatum in the preparation of official ointments, provided pharmacological tests prove satisfactory. We feel this suggestion to be worthy of the consideration of the official revision committees.

SUMMARY

1. A new ointment base consisting essentially of saturated hydrocarbons is reported. It has high stability over a wide range of temperature.

2. This base, Jelene 50W, has been substituted for the bases of 13 official ointments and the new and the official preparations have been studied under various conditions.

3. Jelene 50W allows the preparation of physically superior ointments in most of those studied.

4. Jelene 50W has certain limitations in our experience which have been discussed.

5. Ointments made with Jelene 50W are soft, easily spread, and easily compounded with other substances.

6. The advantages of Jelene 50W for the prescription pharmacist have been demonstrated to our satisfaction and we recommend this substance to the official revision committees for their consideration.

The Effect of Iodides on Phenobarbital Narcosis*

By JOHN C. KRANTZ, Jr., and MARY JANE FASSEL

The narcosis produced in the white rat by phenobarbital sodium is prolonged and intensified by the parenteral or oral administration of iodides. The observation was made with sodium iodide and Organidin. The intraperitoneal injection of solutions of sorbitol, glycerin, and sucrose also prolongs and intensifies the narcosis produced by phenobarbital sodium.

MANY investigators have shown that the intraperitoneal injection of various substances will prolong the narcosis produced by barbituric acid derivatives. Starkenstein and Weden (1) demonstrated that the intraperitoneal injection of cholesterol in rabbits increased the rapidity of onset and duration of sleep produced by intravenous injections of barbital sodium. Farson, *et al.* (2), showed that the intravenous or intraperitoneal injection of cholesterol in the rabbit and the dog intensified the anesthetic action of Pentothal sodium. Other investigators reported that acetylcholine and ergotamine tartrate (3), certain antihistaminic drugs (4), and thiamine hydrochloride (5), respectively, prolonged the narcosis produced by barbituric acid derivatives. Of special interest is the recent work of Wooster and Sunderman (6) who found that certain nitrates and nitrites upon intraperitoneal injection prolonged the anesthesia produced by Pentothal sodium.

For some time we have been interested in the pharmacology of a substance obtained by the interaction of glycerin and iodine, known as Organidin.[1] The product contains 2.5 per cent of iodine bound with glycerin or its products of oxidation. The iodinated compounds have not been identified. There is no free iodine or inorganic iodide present in the preparation. The product elicits antithyroid effects. It was called to our attention by certain clinical observers that when Organidin was administered to patients who were simultaneously taking phenobarbital, the sedation produced by phenobarbital was intensified and prolonged. We decided to subject this observation to critical studies.

EXPERIMENTAL

Young white male Wistar rats were used in these experiments. Many of the various agents were injected intraperitoneally and others were administered orally. Throughout this study, injections of phenobarbital sodium were made intraperitoneally. The term *narcosis* as used herein is the absence of the righting reflex and the presence of a syndrome comparable to light surgical anesthesia.

Although intraperitoneal injections of Organidin produce no demonstrable effect upon the rats, the data in Table I show that this substance lengthens significantly the narcosis produced by phenobarbital sodium. The same condition was seen to prevail with an equivalent amount of sodium iodide injected intraperitoneally. However, the effect was not so pronounced as that with Organidin. The *P* value indicating the statistical difference between the mean of the control rats during narcosis and that of the experimental animals is significant.

TABLE I.—PROLONGATION OF NARCOSIS OF PHENOBARBITAL SODIUM WITH IODIDES

	No. of Rats	Onset of Narcosis, (Min. After Injection)	Duration of Narcosis, (Min.)	*P* Value
Control narcosis (12 mg./100 Gm. phenobarbital sodium)	12	39	103 ± 10	...
Organidin (0.25 cc./100 Gm. of rat)	12	21	226 ± 21	<0.001
Organidin (0.56 cc./100 Gm. of rat)	12	10	367 ± 10	<0.001
NaI I_2/100 Gm. of rat = 0.56 cc. of Organidin	12	21	180 ± 15	<0.001

Since Organidin is obtained by the interaction of iodine and glycerin, we were concerned with the effect of various substances such as glycerin and sorbitol upon the prolongation of the narcosis produced by phenobarbital sodium. These data are shown in Table II.

It is clear from the data in Table II and their statistical analysis that sorbitol, glycerin, and sucrose each lengthen the narcosis produced by pheno-

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[1] Marketed by Henry K. Wampole and Co., Philadelphia, Pa.

TABLE II.—PROLONGATION OF NARCOSIS BY THE INTRAPERITONEAL INJECTION OF VARIOUS AGENTS

	No. of Rats	Onset of Narcosis, (Min. After (Injection)	Duration of Narcosis, (Min.)	*P* Value
Control narcosis (12 mg./100 Gm. phenobarbital sodium)	12	24	136 ± 6	....
NaCl (equivalent to NaI dose)	12	24	145 ± 12	>0.5
NaBr (equivalent to NaI dose)	12	20	140 ± 8	>0.6
Glycerin (0.56 cc. of 50% w/v solution/100 Gm.)	8	16	333 ± 22	<0.001
Glycerin *a* + NaI *b* (*a*) 0.56 cc. of 50% w/v solution/100 Gm. (*b*) I_2/100 Gm. of rat = I_2 in 0.56 cc. of Organidin	8	16	356 ± 16	<0.001
Sucrose (0.56 cc. of 50% w/v solution/100 Gm.)	9	18	261 ± 19	<0.001
Sorbitol (0.56 cc. of 50% w/v solution/100 Gm.)	8	12	269 ± 9	<0.001

barbital sodium. However, it is of special interest that this does not hold true with either sodium chloride or sodium bromide.

In order to determine whether or not the effect was due to the intraperitoneal injection of a substance with high osmotic pressure, the Organidin was given orally to fasted rats thirty minutes prior to administration of phenobarbital sodium. Although there was slight prolongation of the narcosis produced by phenobarbital sodium, the effect of Organidin upon oral administration was not nearly as marked as that upon intraperitoneal injection.

To study further the effect of the intraperitoneal injection of Organidin upon the narcosis of phenobarbital sodium, we administered a non-narcotic dose of phenobarbital sodium (6 mg./100 Gm. of animal). Intraperitoneally this dose narcotized none of the animals. When, however, this non-narcotic dose was given plus Organidin (1.7 cc./100 Gm.), all of the animals were narcotized, indicating that the threshold for narcosis is diminished by the presence of the organic iodide.

The experiment was repeated on fasted rats, using 9 mg./100 Gm. of phenobarbital sodium. In this experiment four out of nine animals were narcotized. However, when the Organidin was administered orally thirty minutes before the phenobarbital sodium, all of the animals were narcotized, indicating that the effect of Organidin in intensifying the narcosis of phenobarbital is also exhibited upon oral administration.

The problem was further investigated by the oral administration of sodium bromide and sodium iodide (six times the intraperitoneal dose) respectively, to fasted rats, followed in twenty minutes by an intraperitoneal injection of 9 mg./100 Gm. of phenobarbital sodium. All of the rats were narcotized, indicating that sodium iodide and sodium bromide when administered orally potentiate the narcosis produced by phenobarbital sodium.

Another series of animals was fed 0.5% sodium iodide in the drinking water for a period of seven days. Prior to the administration of sodium iodide, 9 mg./100 Gm. of phenobarbital sodium produced narcosis in none of 10 animals. After sodium iodide the same dose of phenobarbital sodium elicited narcosis in six of the 10 animals. The average duration of narcosis of the six animals was seventy-five minutes. Thus the presence of iodide in the tissues appears to lower the threshold for phenobarbital sodium narcosis.

DISCUSSION

From the data in Tables I and II it is apparent that substances of the sugar alcohol type, and also the disaccharide sucrose, when injected intraperitoneally into the rat will prolong the narcosis produced by phenobarbital sodium injection. Inorganic iodides produce a similar response, and when the iodide and a sugar alcohol such as glycerin are combined, the potentiation of narcosis is increased. It is our opinion that the effects of these substances exhibiting high osmotic pressure injected into the peritoneal cavity, are due to the osmotic pressure changes in the animal. Thus one may speculate that fluid is drawn from the extracellular spaces, tissues, and the blood, and that the concentration of the narcotic agent in the circulating blood is thereby increased. Through this mechanism the barbiturate concentration in the brain may be increased.

Since glycerin, sorbitol, and sucrose upon oral administration are metabolized and ultimately find their way into carbon dioxide and water, one would not expect them to exert the foregoing effect upon oral administration. We do not have any adequate explanation for the effect of the iodide ion in potentiating the action of phenobarbital sodium. It is true that the iodides are used for their vascular action and to a limited extent they may emulate the action of nitrites and nitroglycerin. Wooster and Sunderman (6) hold the view that vasodilators of the nitrite-nitrate series produce increased cerebral blood supply and this in turn may be related to a greater amount of barbiturate reaching the brain. This condition may prevail with iodides. There occurs, however, another possibility. Iodides have been shown to decrease oxygen uptake of tissues through inhibition of the pituitary-thyroid axis. Organidin shares this action. This effect may render the organism more susceptible to depression by barbiturates. It appears that the mechanism

must be different from that of depression *per se* owing to the fact that the bromide ion, which is known to exert a depressing effect upon the cerebral cortex, is not as efficient as the iodine ion in potentiating the action of phenobarbital sodium.

It is well established that phenobarbital is excreted in the urine unchanged. We therefore tested the synergistic action of Organidin with a barbiturate which is readily metabolized. Maynert and van Dyke (7) showed that pentobarbital is converted into a hydroxyl derivative and excreted. With pentobarbital the effect of Organidin was similar to that observed with phenobarbital (10 animals). Thus the synergism appears to obtain with the barbiturates which are mainly metabolized, as well as with those which are chiefly excreted.

SUMMARY

1. It has been shown that in the white rat the narcosis of phenobarbital sodium is prolonged by the intraperitoneal administration of glycerin, sucrose, sorbitol, inorganic iodides, and Organidin.

2. The oral administration of sodium iodide, sodium bromide, or Organidin increases the length of narcosis produced by the intraperitoneal injection of phenobarbital sodium.

3. The mechanism of the potentiation is discussed.

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Determination of Nicotinic Acid in Pharmaceutical Products*,†

By ALEXANDER MUELLER and SERECK H. FOX‡

The quantitative chemical determination of nicotinic acid employing ammonia and cyanogen bromide is described. The procedure is rapid and simple. Data are easily reproducible with reagents which are stable. Typical analytical data are presented which indicate that the method is exceptionally precise in its application to pharmaceutical preparations. Nicotinamide is determined as nicotinic acid after hydrolysis of sample.

COMPREHENSIVE literature reviews of nicotinic acid colorimetric procedures employing various aromatic amines are available (1, 2). These indicate the complicating factors involved. A brief description of the colorimetric determination of nicotinic acid employing ammonia and cyanogen bromide was reported by the authors a few years ago (3).[1] Since the prior publication (3), thousands of analyses with the ammonia method have been performed by several analysts in this laboratory. It has been successfully applied to a variety of pharmaceutical mixtures. The relatively simple procedure has produced consistent results with a minimum of inexpediency. The ammonia method has eliminated many objections inherent in the use of organic amines. The reagents are stable, readily available, and reproducible. The color obtained exhibits a comparatively large absorption constant; therefore small variations in concentration cause an appreciable change in absorbancy, resulting in high sensitivity in the step of color estimation.

When nicotinic acid is reacted with cyanogen bromide in the presence of ammonia, a bright yellow color is produced. The color is proportional to concentration when plotting absorbancy at the maximum wave length against concentration of nicotinic acid. The wave length maximum is at 408 mμ, reaching its highest intensity at two to two and one-half minutes after addition of reagent. The extinction coefficient $E^{1\%}_{1cm}$ is 4,300 to 4,600 (dependent on a given set of conditions). The wave length maximum for

* Received August 20, 1951, from the R. P. Scherer Corp., Gelatin Products Div., Detroit, Mich.

† Since the preparation of this manuscript, Sweeney and Hall have reported on the use of sulfanilic acid and ammonium hydroxide in the analysis of nicotinic acid: *cf. J. Assoc. Offic. Agr. Chemists*, **34,** 380(1951), and *Anal. Chem.*, **23,** 983 (1951).

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[1] This method, substantially in its present form was considered by the then active Vitamin Committee of the American Drug Manufacturer's Association and by various members of the Nicotinic Acid Sub-Committee of that Committee. In addition, members of the Food and Drug Administration have been given the opportunity of private trials with it.

nicotinamide is at 398 mμ, reaching its maximum intensity at six minutes, with a slightly lower extinction coefficient. Since the rate of reaction with nicotinamide is slower than with nicotinic acid, it is more expedient to hydrolyze the amide to the acid, then proceed with the analysis as nicotinic acid. Either acid or alkali hydrolysis may be employed. If acid is used, the solution after hydrolysis should be adjusted to a *p*H above 2.0. Slightly acid or a basic medium does not interfere with the color development. Generally, with pharmaceutical preparations, the increment procedure is unnecessary. In cases where the nature of the contaminant causes high or low results the increment procedure can be readily adapted to the procedure described herein without great inconvenience. With colored solutions a blank is prepared and the absorbancy value of the blank subtracted from the total.

EXPERIMENTAL

Reagents and Apparatus

U. S. P. Reference Standard Nicotinic Acid.—A stock solution containing 200 μg./cc. is prepared in distilled water and stored in refrigerator. Remove an aliquot by pouring 10–15 cc. into a beaker, allow to come to room temperature, then make a dilution containing 3–5 μg./cc. for standardization. Dilute solutions (3–5 μg./cc.) of nicotinic acid are not stable at room temperature for more than twelve hours.

Cyanogen Bromide Reagent.—A 10% solution is prepared by dissolving 50 Gm. of crystalline cyanogen bromide (Eastman) in 500 ml. of distilled water. To hasten solution the mixture is shaken in a mechanical shaker in a tightly stoppered bottle (glass stopper). The CNBr solution may be kept in the hood at room temperature when in daily use. No deterioration of this solution has been found at the end of one month. The crystalline CNBr is stored in a refrigerator. Weighing, dispensing of CNBr solution, and rinsing of glassware containing CNBr must be performed in an efficient hood.

Buffered Ammonia Reagent.—To a 1-L. volumetric flask containing about 500 cc. of distilled water add 87.0 Gm. of dipotassium phosphate (K_2HPO_4), reagent grade, and 107.0 Gm. of ammonium chloride, (NH_4Cl), reagent grade. Shake to dissolve the salts, then add 6.7 cc. of concentrated ammonium hydroxide (28%), reagent grade, and make to volume with water. Filter, if a sediment forms on standing.

Sodium Hydroxide Solution, Reagent Grade, 0.5 *N*.

Hydrochloric Acid, Reagent Grade, Concentrated.

Colorimeter.—The Evelyn Colorimeter with 19-mm. standardized test tubes and a double 420 mμ filter was used for most of data here reported. The investigational aspects were accomplished with the Model DU Beckman Spectrophotometer. The double 420 mμ filter in the Evelyn was found to be more satisfactory than a single filter, since the double filter selects a narrower band width and in turn gives $L^{1\%}_{1cm.}$ values comparable to the $E^{1\%}_{1cm}$ values of the Beckman.

Procedure with Pharmaceutical Samples

Place a suitable number (not less than two) of capsules, tablets, etc., in a 1-L. volumetric flask, add about 200 cc. of water and place the flask on a steam bath and swirl at intervals until sample is dispersed. If the niacin is in a fatty base, 3–5 cc. of ethylene dichloride is added as a solvent for the fats. Cool and make to volume (allow for fat solvent) with water and filter an aliquot for hydrolysis (if sample is niacinamide).

Hydrolysis

Pipette a 10-cc. aliquot of the above filtrate into a volumetric flask (100–250 cc.). The size of the flask selected should be such that when the solution is made to volume it will contain a concentration of 3–5 μg. of niacinamide per cc. Add 10 cc. of concentrated hydrochloric acid and digest on a hot plate for twenty to thirty minutes. The heat must be so adjusted that at the end of the hydrolysis period approximately 2 cc. of solution remains. Boiling to dryness must be avoided. Cool and add 40–50 cc. of water. Add three to five pellets of potassium hydroxide and make to volume. This solution having a *p*H between 2 and 12 is employed for the colorimetric measurements. If an alkaline hydrolysis is preferred the procedure is as follows: add 10 cc. of 0.5 *N* sodium hydroxide solution and digest as above, observing the same precautions. After cooling, the flask is made to volume with water and requires no adjustment of *p*H.

Color Measurement

The technique here described is intended for the Evelyn Colorimeter and may be adapted to a different instrument if desired. To a series of matched colorimeter tubes add the following:

	Tube No.				
	1	2	3	4	5
Standard solution nicotinic acid, cc.	..	1	..	..	1
Test solution nicotinic acid, cc.	..	..	1	1	1
Ammonia buffer, cc.	3	3	..	3	3
Cyanogen bromide reagent, cc.	5	5	..	5	5
Distilled water, cc.	2	1	9	1	..

The reagents are dispensed from automatic burettes. The tubes are rubber-stoppered before being placed in the instrument. The concentrations of the nicotinic acid solution are prepared so that their absorbancy is not less than 0.2 and not more than 0.5.

Place tube 1 in the colorimeter and set the instrument for zero absorbancy (100% transmittancy). Place tube 2 in the colorimeter and take the absorbancy reading (*C*) at maximum color intensity between two and two and one-half minutes. Prepare tube 3 and read against tube 1; this is the test solution blank (*B*). Prepare tube 4 and read against tube 1 at maximum color intensity (*A*) between two and two and one-half minutes. Tube 5 is used for the increment procedure (see next page).

Calculations

$$\frac{A - B \times S}{C \times W} = \mu g.\text{ of nicotinic acid per tablet, capsules, Gm., etc.}$$

A = absorbancy of test solution
B = test solution blank
C = absorbancy of standard solution
S = μg. in standard aliquot per cc.
W = fraction of tablet, capsule, Gm., etc., represented in each cc. of final dilution.

In a continuous series of readings lasting several hours the standard solution absorbancy should be rechecked at intervals of one to one and one-half hr.

When the increment procedure is required, prepare tube 5 and take absorbancy reading (T) at maximum color intensity between two and two and one-half minutes. Calculate the result as follows:

$$\frac{A - B \times S}{T - A \times W} = \mu g.\text{ of nicotinic acid per tablet, capsule, Gm., etc.}$$

where T is the absorbancy of test solution plus standard.

DISCUSSION

Data on various types of pharmaceutical capsules are presented in Table I. The data shows the precise performance of the method in the presence of interfering materials such as iron, liver, and yeast, and comparison of results employing either acid or alkaline hydrolysis. It is evident from examination of the data that either acid or alkaline hydrolysis may be used with equal accuracy or pre-

TABLE I.—RESULTS ON VARIOUS TYPES OF MIXTURES WITH ACID AND ALKALINE HYDROLYSIS

Description and No.	Mg. Niacin Theory	HCl Hydrolysis	Recovery, %	NaOH Hydrolysis	Recovery, %
Multivitamin capsules					
46975	10.5	10.73	102.2	10.50	100.0
		10.73	102.2	10.67	101.6
46708	18.0	18.12	100.6	17.72	98.4
		18.12	100.6	17.99	99.9
46507	20.0	20.34	101.7	20.06	100.3
		20.34	101.7	20.27	101.3
46670	20.0	20.26	101.3	20.00	100.0
		20.00	100.0	20.00	100.0
47142	22.0	21.94	99.7	21.94	99.7
		22.21	100.9	21.94	99.7
46573	25.0	25.01	100.0	25.40	101.6
		25.40	101.6	25.81	103.2
47411	25.0	24.88	99.5	24.88	99.5
		25.26	101.0	25.26	101.0
47182	40.0	38.62	96.5	40.50	101.2
		39.13	97.8	40.77	101.9
46608	165	162.91	98.7	162.91	98.7
		162.91	98.7	164.90	99.9
47180	150	147.05	98.0	148.80	99.2
		148.03	98.7	145.08	96.7
Multivitamin capsules with ferrous sulfate					
47018	7.7	7.63	99.0	7.73	100.4
		7.68	99.7	7.68	99.7
47135	7.7	7.99	103.7	7.84	101.8
		7.94	103.7	7.89	102.4
Multivitamin capsules with ferrous gluconate and liver conc.					
46637	12.5	12.30	98.4	12.95	103.6
		12.30	98.4	12.85	102.8
Multivitamin capsules with ferrous sulfate and whole dried liver					
46997	50.0	51.71	103.4	50.91	101.8
		52.09	104.2	51.71	103.4
47445	50.0	48.70	97.4	48.15	96.3
		48.70	97.4	48.77	97.5
Multivitamin capsules with yeast					
47574	11.0	11.16	101.40	11.07	100.6
		11.23	102.1	11.16	101.4
Multivitamin capsules with yeast and liver conc.					
46685	20.0	20.20	101.0	20.27	101.3
		20.00	100.0	20.27	101.3
46686	20.0	19.91	99.5	19.91	99.5
		20.10	100.5	19.78	98.9

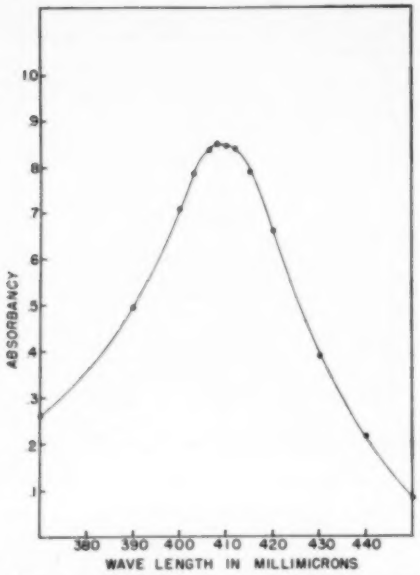

Fig. 1.—Absorption spectrum of nicotinic acid-ammonia-cyanogen bromide reaction product. Concentration 0.000195%; time two and one-half min.

cision. It is preferable, however, to apply the alkaline hydrolysis since it saves a step in the procedure of adding NaOH pellets to neutralize the excess acid.

The 10% cyanogen bromide reagent was observed to be the optimum concentration. Stronger solutions have the tendency to crystallize at room temperature. Weaker solutions, 5% and 7%, freshly prepared, gave equal color response, but the reaction rate was retarded. The weaker reagents required three to five minutes for maximum color development.

The spectrophotometric absorption curve of the reaction product of nicotinic acid-ammonia-cyanogen bromide is shown in Fig. 1. The curve represents the condition of the reaction at exactly two and one-half minutes. In order to obtain a constant time curve, a separate reaction mixture (concentration constant) was prepared for each wave-length setting and clocked at two and one-half minutes.

Nicotinamide reacts with ammonia and cyanogen bromide at a slower rate and produces its absorption peak at a lower wave length, as previously indicated. By observing whether the absorbancy of the reaction mixture declines or continues to increase at 408 mμ after three minutes, an indication is obtained if nicotinamide is present. This technique may also be employed to determine completeness of hydrolysis of the amide. On equimolar quantities of nicotinic acid and nicotinamide, the latter produces about three-fourths of the absorption intensity at 408 mμ in two and one-half to three minutes, as compared to the acid.

Elvehjem and Teply (4) have pointed out the difficulties which can arise with colorimetric methods for nicotinic acid employing cyanogen bromide. Ammonia, as well as the previously used aromatic amines, gives a color with pyridine and certain of its derivatives. Fortunately, little difficulty is encountered in pharmaceutical mixtures, due to the absence of pyridine compounds.

SUMMARY

1. The ammonia-cyanogen bromide method for the quantitative determination of nicotinic acid is described.

2. Data on representative pharmaceutical mixtures employing both acid and alkaline hydrolysis are presented.

3. The procedure is rapid, simple, and enables the operator to obtain accurate data with a minimum of difficulty.

4. The spectrophotometric absorption curve showing the well-defined color is given.

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Studies on the Stabilization of Human Serum Albumin. The Effect of the *p*H, the Stabilizers, and the Albumin*,†

By JOHN H. HINK, JR., and FREDERICK F. JOHNSON

The effect of *p*H on the stability of human serum albumin, as measured by light scattering at elevated temperatures, is reported. The study includes the relationship between *p*H and the various albumin stabilizers, and also includes the effectiveness of the stabiiizers on relatively native albumin and relatively denatured albumin.

A SOLUTION (25 per cent) of human serum albumin becomes more viscous and exhibits an easily measurable increase in light scattering upon exposure to heat for extended periods of time. Several stabilizers have been found to be effective in minimizing the increase in turbidity. Whereas 25 per cent albumin in 0.15 *M* NaCl can be heated only a few minutes at 57° before a visible turbidity appears, the same albumin in 0.30 *M* NaCl can be heated approximately twenty-four hours before a visible turbidity appears (1). The addition of 0.04 *M* acetyl tryptophane in place of the NaCl allows the albumin solution to be heated at 60° for ten hours, and the addition of 0.04 *M* acetyl tryptophane plus 0.02 *M* sodium caprylate allows the solution to be heated at 64° for ten hours before appearance of the first visible turbidity (2). Luck and his co-workers (3–6) have used sodium caprylate and related compounds to prevent any change in light scattering of bovine albumin and human albumin solutions under conditions which would otherwise cause severe denaturation.

This study, made chiefly with acetyl tryptophane and sodium caprylate, was undertaken in order to determine the optimum *p*H and the optimum mixture of stabilizers for minimizing denaturation at 57° and 60° as measured by light scattering.

METHODS

Turbidimetric methods were used as a measure of denaturation at elevated temperatures. A special photoelectric tyndallometer (1) was used which measures light scattered at an angle of 90°. This tyndallometer also measures transmitted light, by which means any decrease in percentage transmission of light due to an increase in color of the albumin can be measured and applied as a correction to the measurements of scattered light. The turbidity values are expressed in terms of "nephelometric units." A unit is approximately 1% of the turbidity of a standard of absolute turbidity 0.0193.

Hydrogen-ion determinations of the albumin solutions were made on 1.0% albumin prepared by diluting the 25% albumin solutions with 0.02 *M* sodium chloride free of carbon dioxide. The *p*H of the diluted solutions was then measured with a glass electrode standardized against three buffers of different *p*H.

RESULTS

Effect of *p*H on Stabilization.—Early experiments with human serum albumin prepared by the methods of Cohn indicated a maximum heat stability at *p*H 6.8. Scatchard (1), studying 25% albumin dissolved in 0.3 *M* NaCl, reported the maximum stability at 57° to be at *p*H 6.75 and the maximum stability at 50° to be at a slightly higher *p*H value.

Solutions of albumin (25%) were prepared in 0.3 *M* NaCl and were adjusted to different *p*H values and filtered by the same method as used by Scatchard. These albumin solutions were maintained at 50° and 57° and nephelometric measurements were made daily as previously described. Two types of albumin were used in these experiments: the so-called "stable" albumin was prepared without incident by method 6 of Cohn (7); the so-called "unstable" albumin was prepared in the same manner, but was slightly denatured by exposure to 40% ethanol at 0°.

In Fig. 1, the days at 50° and 57° required for an increase of 20 nephelometric units are plotted against *p*H. The "unstable" albumin being the more stable at 50° is not too uncommon. When albumin is slightly denatured by alcohol above 0°, traces of globulin impurities are denatured sufficiently to be removed from solution, and the resulting more pure albumin can show a more favorable stability over a short period of time. At 57°, the "unstable" albumin appeared approximately as stable as the "stable" albumin, but, as will be seen in the subsequent data, this was true only for albumin in a diluent of 0.3 *M* NaCl, in which albumin is not particularly stable anyway. The 50° stability of our albumin was not quite as great as that reported by Scatchard (1), but the 57° stability was a little greater. The *p*H of maximum stability in 0.3 *M* NaCl appeared to be between 6.7 and 6.8 at 57°, and between 6.9 and 7.0 at 50°. This appeared to agree very closely with Scatchard's data, and served as a check on the technique for subsequent work.

Similar experiments were conducted with "salt poor" albumin using acetyl tryptophane and sodium caprylate as stabilizers singly and mixed in various proportions. It was known from previous work that the stabilizers being used would protect albu-

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† Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.

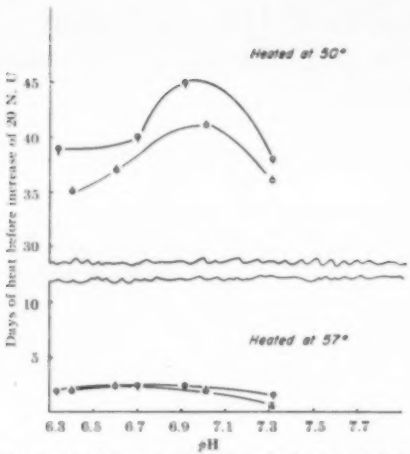

Fig. 1.—Stability of 25% albumin in 0.3 *M* NaCl. ◊ "Stable" albumin, ♀ "unstable" albumin.

min against 50° denaturation for a length of time impractical to be measured; so the 50° heating test was discontinued. One-half of the samples were heated only at 57°. The other samples were heated at 60° for ten hours prior to the 57° heating, and the time for an increase of 20 nephelometric units was computed from the start of the 57° heating. The data from these experiments are shown in Figs. 2, 3, 4, and 5. It is apparent from these figures that the *p*H for best stability with acetyl tryptophane or sodium caprylate as stabilizers is higher than the *p*H for best stability of albumin in 0.3 *M* NaCl. The optimum *p*H range for high temperature stability in the presence of these organic stabilizers seems to be generally between 7.1 and 7.3.

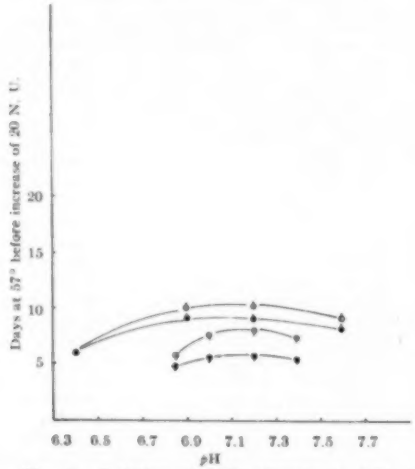

Fig. 2.—Stability of 25% albumin in 0.04 *M* sodium acetyl tryptophane. ◊ "Stable" albumin, ♀ "unstable" albumin. Solid symbols indicate sample heated ten hours at 60° prior to study.

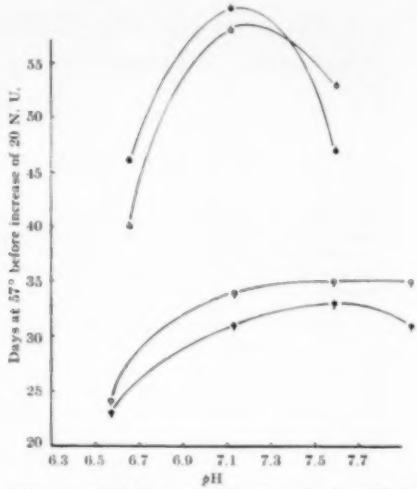

Fig. 3.—Stability of 25% albumin in 0.02 *M* sodium acetyl tryptophane plus 0.02 *M* sodium caprylate. ◊ "Stable" albumin, ♀ "unstable" albumin. Solid symbols indicate sample heated ten hours at 60° prior to study.

In Fig. 5, the results indicate that with a stabilizing mixture of 0.1 *M* acetyl tryptophane and 0.04 *M* sodium caprylate, the stability of the albumin exceeds the practical limits of the 57° stability test. Samples not previously heated at 60°, and at the lower *p*H values, gelled before showing an increase of 20 nephelometric units. With the gel being less opaque at lower *p*H value, the "stable" albumin at 57° showed no *p*H of maximum stability as measured by light scattering.

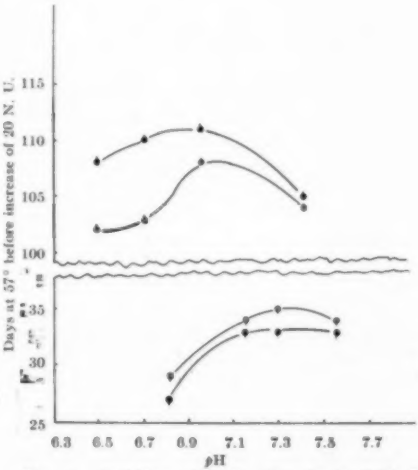

Fig. 4.—Stability of 25% albumin in 0.04 *M* sodium caprylate. ◊ "Stable" albumin, ♀ "unstable" albumin. Solid symbols indicate sample heated ten hours at 60° prior to study.

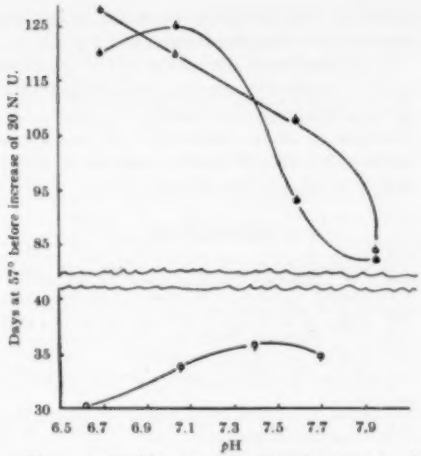

Fig. 5.—Stability of 25% albumin in 0.01 *M* sodium acetyl tryptophane plus 0.04 *M* sodium caprylate. ◊ "Stable" albumin. Solid symbols indicate sample heated ten hours at 60° prior to study. ♀ "Unstable" albumin with and without prior heating.

In some instances, the albumin samples which had been heated at 60° for ten hours required a longer time at 57° before showing the increase of 20 nephelometric units. This is not due to any stabilizing effect of the 60° heat, but is due to the fact that the initial nephelometric reading is usually two to five units higher after the 60° heat, and this initial reading is taken as the starting point for the measured increase of 20 nephelometric units.

Effect of Stabilizer Mixtures.—In order to determine the most effective practical mixture of acetyl tryptophane and sodium caprylate, solutions of the "unstable" albumin used in the preceding experiments were prepared containing different amounts of acetyl tryptophane and sodium caprylate. The solutions were all adjusted to *p*H 7.2, and after an initial nephelometric reading they were subjected to 57° heat. Daily readings were made, and the number of days required for an increase of 20 nephelometric units are recorded in Table I.

TABLE I.—ALBUMIN STABILITY AT 57° WITH ACETYL TRYPTOPHANE AND SODIUM CAPRYLATE STABILIZERS

Molarity of Stabilizer in 25% Albumin	Initial N. U.[a]	Days at 57° Before Increase of 20 N. U.
None	9	<1
0.04 *M* AcT	9.5	9–10
0.08 *M* AcT	13	18
0.01 *M* AcT 0.02 *M* SC	10	20
0.01 *M* AcT 0.03 *M* SC	8.5	25
0.01 *M* AcT 0.04 *M* SC	9	30
0.02 *M* AcT 0.02 *M* SC	9	26
0.02 *M* AcT 0.03 *M* SC	11	26
0.02 *M* AcT 0.04 *M* SC	9	29–30
0.03 *M* AcT 0.02 *M* SC	8.5	25
0.03 *M* AcT 0.03 *M* SC	8.7	34
0.03 *M* AcT 0.04 *M* SC	14	32
0.04 *M* AcT 0.02 *M* SC	13	26
0.04 *M* AcT 0.04 *M* SC	9	26
0.01 *M* SC	9.6	2
0.02 *M* SC	9.5	14
0.03 *M* SC	9.0	24–25
0.04 *M* SC	8.5	25
0.08 *M* SC	13	25

[a] All results are the average of two samples. AcT = acetyl tryptophane. SC = sodium caprylate. N. U. = nephelometric units.

Effect of Degree of Albumin Denaturation on Stabilization.—Experiments previously conducted on a variety of albumin solutions indicated that different preparations of albumin required different stabilizer mixtures for achieving optimum stability. An attempt was made to clarify the relationship by studying the stabilization of three different albumin preparations: stable albumin carefully prepared by method 6 of Cohn (designated as 160–137); unstable albumin described in the previous experiments (designated as 262-1-1); very unstable albumin fractionated from ultraviolet irradiated unstabilized plasma (two preparations desig-

TABLE II.—STABILITY OF DENATURED AND UNDENATURED ALBUMIN AT 57° AND *p*H 7.2 WITH ACETYL TRYPTOPHANE AND SODIUM CAPRYLATE STABILIZERS

Type of Albumin		Molarity of Stabilizer in 25% Albumin	Initial N. U.[a]	Days at 57° Before Increase of 20 N. U.
"Unstable"	160–133	0.04 *M* AcT	6	1-2
"Unstable"	160–139	0.04 *M* AcT	6	1-2
"Unstable"	160–133	0.02 *M* AcT + 0.02 *M* SC	6	3-4
"Unstable"	160–139	0.02 *M* AcT + 0.02 *M* SC	6	5
"Unstable"	160–139	0.03 *M* SC	6	1-2
"Slightly Unstable"	262–1–1	0.04 *M* AcT	9	8
"Slightly Unstable"	262–1–1	0.02 *M* AcT + 0.02 *M* SC	8	34
"Slightly Unstable"	262–1–1	0.04 *M* SC	10	34
Stable	160–137	0.04 *M* AcT	8	10
Stable	160–137	0.02 *M* AcT + 0.02 *M* SC	8	58
Stable	160–137	0.04 *M* SC	8	110

[a] All results are the average of two samples. AcT = acetyl tryptophane. SC = sodium caprylate. N. U. = nephelometric units.

nated as 160–133 and 160–139). Many experiments were conducted, but only those which are strictly comparative and which employed a total stabilizer concentration in the region of 0.04 *M* are reported here. The data are summarized in Table II. The greater the degree of albumin denaturation, the greater is the relative stabilizing effect of acetyl tryptophane. When albumin denaturation is held at a minimum, sodium caprylate alone effects better stabilization than does acetyl tryptophane or a mixture of the two.

SUMMARY

1. The results of earlier investigators concerning the high temperature stability of 25 per cent human serum albumin in 0.3 *M* sodium chloride are confirmed: as measured by light scattering, the greatest stability at 57° is at *p*H 6.7 to 6.8; greatest stability at 50° is at *p*H 6.9 to 7.0.

2. At 57°, the optimum stability of albumin stabilized with acetyl tryptophane or sodium caprylate is within the *p*H range of 7.1 to 7.3.

3. Undenatured albumin at 57° is stabilized more effectively by sodium caprylate than by acetyl tryptophane or by a mixture of the two; as denaturation of the albumin is increased, stabilization is better effected by a mixture of sodium caprylate and acetyl tryptophane.

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Cellulose Acetate Phthalate as an Enteric Coating Material*

By C. J. MALM, J. EMERSON, and G. D. HIATT†

Cellulose acetate phthalate is soluble in buffer solutions at *p*H values as low as 6. It has therefore found a use in the enteric coating field. Addition of plasticizers improves the water resistance of the ester. Enteric coatings of such compositions are more effective than when the ester alone is used. Aqueous solutions can be prepared by treating the acetate phthalate with bases such as sodium hydroxide or triethanolamine.

IN RECENT years considerable interest has been shown in cellulose acetate phthalate as an enteric coating material. This ester and its salts have been disclosed in patents by Malm and Waring (5) and have been more fully described by Malm and Fordyce (4), who show that one carboxyl of the phthalic acid is combined with cellulose, and the other is free for further reactions. The resulting polymeric acid forms water-soluble alkaline salts.

Its use as an enteric coating was first described by Hiatt (1) and further tested by Hodge (2, 3) who found in animal feeding experiments that cellulose acetate phthalate "seems to be remarkably inert as a compound of the diet" (2). There is no evidence in the laboratory and plant handling of this product to indicate any allergenic or sensitizing effects. This ester, in addition to being soluble in dilute alkali, is readily soluble in a number of common organic solvents. Additional studies have been made to make available information which should lead to a better understanding and a wider use of this material.

CHARACTERISTICS OF ESTER

Composition.—The type of cellulose acetate phthalate chosen for this study is one which is made by treating a cellulose acetate of 30–32% acetyl content with phthalic anhydride and a tertiary organic base, such as pyridine. The reaction does not completely esterify the available hydroxyl groups. The resulting product analyzes 34–38% combined phthalyl. The particular material used in this study showed a phthalyl content of 35.2%

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† The authors wish to acknowledge the comments of Dr. Harold Hodge of the University of Rochester School of Medicine and Dentistry, who read the manuscript.

and an acetyl value of 17.8%. These values indicate that an average glucose unit would contain approximately 0.8 phthalyl, 1.4 acetyl, and 0.8 hydroxyl groups.

Solubility.—Because many uses of cellulose esters involve solvents, considerable emphasis is placed on solubility in organic solvents. Table I lists solubilities of the cellulose acetate phthalate in single organic solvents and some mixtures. These tests were run by mixing 1.0 Gm. of cellulose ester with 9 Gm. of solvent and observing after twenty-four hours at room temperature. Alcohols alone are not solvents, nor are chlorinated hydrocarbons, but mixtures of these are. The same is true of alcohol-aromatic hydrocarbon combinations. Ketones are quite active solvents, as are the ether-alcohols and the acetates of these compounds (methoxyethyl alcohol and methoxyethyl acetate). Cyclic ethers are good solvents, whereas straight chain ethers are poor.

Viscosity.—The phthalate derivative shows the same viscosity trend as acetone-soluble cellulose acetates. Increasing the ester concentration in a solvent such as acetone causes the viscosity to increase as a logarithmic function. Data on the cellulose acetate phthalate dried to a moisture content of 1.3% and dissolved in acetone containing 0.22% moisture are shown in Fig. 1.

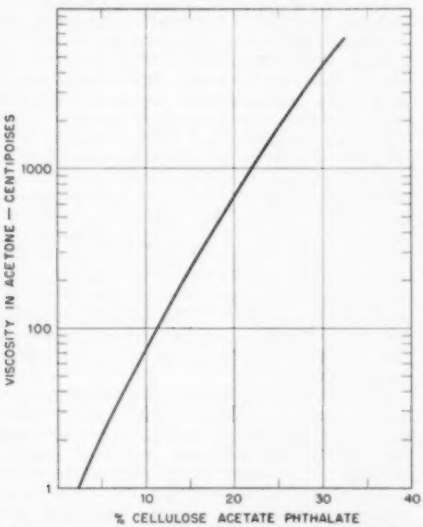

Fig. 1.—Viscosity-concentration curve for cellulose acetate phthalate in acetone.

A 15% solution of this ester in acetone has a honey-like consistency and is a good solution for test coatings. The viscosity is influenced by the purity of the acetone, as for example, the amount of water present. Of interest also is the change in viscosity of 15% solutions in which ethyl alcohol is progressively substituted for acetone. The data in Fig. 2, curve 1, were obtained using the previously dried cellulose ester and acetone of low water

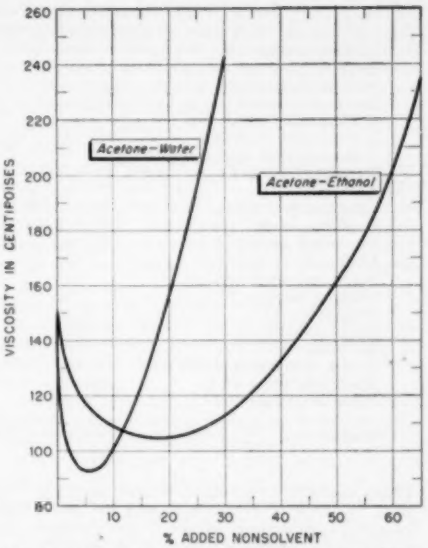

Fig. 2.—Influence of solvent composition on viscosity.

content and adding distilled water. A sample of special low-water acetone (0.04%) was used to make up the solution which showed the viscosity of 150 c.p.s. Absolute ethyl alcohol was used as the nonsolvent in curve 2. Water increases the solvent power of acetone up to a content of about 7%. Unfortunately, this type of solution gives dull, uneven coatings since the water concentrates during solvent evaporation and tends to precipitate the acetate phthalate. To obtain good coatings, only about 3% of water can be tolerated in the original solution. Ethyl alcohol gives a broader low-viscosity range, producing solutions which can be used to give satisfactory coatings. The 80:20 acetone:ethanol mixture allows the highest solids for a given viscosity. Extremely high amounts of alcohol (70%) tend to give poor coatings.

Plasticizer Compatibility.—The addition of plasticizers to cellulose ester films generally improves flexibility and, depending on the plasticizer, may increase water resistance. Tests were made on a limited series of materials which should not be objectionable from a toxicity standpoint. A stock solution was made up to contain 15% acetate phthalate in acetone. To 100-Gm. portions were added 1.5, 3.0, and 4.5 Gm. of plasticizer. Films were coated and dried to give tabs 0.003 in. thick and containing 10, 20, and 30% added plasticizer. Compatibility was recorded after an aging period to note the occurrence of blushing or other forms of plasticizer separation. The results are shown in Table II.

Compatibilities with Other Cellulose Esters.—Attempts are sometimes made to secure special effects by blending different cellulose esters. Tests were made to measure the compatibilities of the acetate phthalate ester by mixing it in acetone solution with 10, 50, and 90% of the test ester.

TABLE I.—SOLUBILITIES OF A TYPICAL CELLULOSE ACETATE PHTHALATE[a]

Methyl alcohol	−	Diethyl ether	−
Ethyl alcohol	−	Diisopropyl ether	−
Propyl alcohol	−	Dioxane	+
Isopropyl alcohol	−	Ethylene formal	+
β-Methoxyethyl alcohol	+	Benzene	−
β-Ethoxyethyl alcohol	+	Benzene:methanol 2:1	−
β-Butoxyethyl alcohol	−	Benzene:methanol 1:1	+
Diacetone alcohol	+	Toluene	−
Acetone	+	Toluene:methanol 2:1	−
Ethyl methyl ketone	+	Toluene:methanol 1:1	+
Cyclohexanone	+h	Cyclohexane	−
Methanol:acetone 1:1	+	Methylene dichloride	−
Methanol:acetone 2:1	+	Ethylene dichloride	−
Methanol:acetone 3:1	+	Ethylene dichloride: methanol 4:1	+
Ethanol:acetone 1:1	+	Propylene dichloride	−
Methyl acetate	+	Propylene dichloride: methanol 4:1	+
Ethyl acetate	−		
Ethyl lactate	+		
Ethylene glycol monoacetate	+		
Methoxyethyl acetate	+		
Ethoxyethyl acetate	+		

[a] Solubility code: + = soluble, − = insoluble, h = hazy.

These solutions were coated on glass plates to thin films, the solvent evaporated, and the dried films allowed to age for ten days under room temperature conditions. Cellulose acetates of 38 and 40% combined acetyl are found to be compatible at 10 and 50% levels but incompatible (opaque) at 90%. Mixed cellulose esters, such as cellulose acetate butyrate, ranging in butyryl from 16 to 40%, are not compatible even at a 10% level. This is not unusual, since cellulose esters of different kinds and degrees of substitution generally show quite limited compatibility with one another.

EXPERIMENTAL

For testing new batches of cellulose acetate phthalate for enteric coatings it is desirable to coat small samples of tablets to note the smoothness of coating and the efficiency of coverage. For this purpose the following procedure is used.

A one-liter metal beaker is charged with about 400 commercial 10-grain salt (sodium chloride) tablets. (For convenience, 270 Gm. of tablets are used.) The beaker is tilted at a 45° angle and rotated to simulate the action of a commercial coating pan. A gentle blast of air is used to remove salt dust. This is important since otherwise this dust may get into the coating and act as a source of pinholes. All coating operations are done at room temperature.

TABLE II.—PLASTICIZER COMPATIBILITY IN COATED FILMS

Plasticizer	Compatibility Limit, %
Dimethyl phthalate	30[a]
Diethyl phthalate	30[a]
Ethyl phthalyl ethyl glycollate (Santicizer E15)	30[a]
Butyl phthalyl butyl glycollate (Santicizer B16)	10
Triacetin	30[a]
Tripropionin	30[a]
Dibutyl tartrate	30[a]
Tributyl citrate	10

[a] Highest level tested.

A solution (15%) of standard cellulose acetate phthalate is made up in acetone. In general it is desirable that the water content of the acetone be no higher than 0.5%, since water in the solvent tends to induce blushing or opacity in the coating. A good grade of commercial acetone is usually of sufficiently high purity. Lower solid contents may be used in the coating solution, though it is desirable to apply the coating as rapidly as possible.

For test purposes three different coating levels are tried. Tablet samples are taken after the addition of 24, 48, and 96 cc. of solution to the 400 tablets. The first 24 cc. is added in six equal quantities. After the first 4-cc. addition is made, the tablets are tumbled to secure an even distribution of the solution. An air blast is then used to flash off the solvent. When the tablets are apparently dry and free tumbling, the air is shut off and the next addition is made. The addition, tumbling, and drying require about two to three minutes so that the 24-cc. level coating can be applied in about twenty minutes.

(In large tumbling pans it may be necessary to aid tumbling by manual stirring since the wet tablets may bunch and tend to "gallop." In a short time the tablets are uniformly wet and tumbling smoothly. Thereafter an air current speeds the drying without tablet sticking. The use of the air blast at the same time that the solution is being added may cause the phthalate to dry in local concentrations and cause clumping.)

After 24 cc. has been added, the tablets have a smooth, glossy appearance but generally show pinholes in the subsequent acid test solution. This level would, however, be useful for retaining medicaments of a low degree of water solubility. The 48-cc. and 96-cc. levels are built up as already described, except that somewhat smaller additions may be required (2–3 cc.) to prevent tackiness and sticking. The time between additions need not be shortened. The higher levels of application increase the water resistance of the coating.

For laboratory use a small amount of dye (0.2 Gm. of crystal violet per 100 Gm. of solution) may be used to observe coating efficiency. An even, shiny, purple coverage indicates proper application.

Effect of Plasticizer in Coating.—Salt tablets coated with the acetate phthalate plasticized with three of the plasticizers mentioned above were tested at a 48-cc. coating level. Ten tablets with each test coating were immersed in 0.25% HCl solution at 98° F. and held for three hours. At the end of this time, the tablets without obvious flaws were wiped dry and weighed. (In these small-scale coatings, tablets frequently have flaws which would not occur in commercial coating pans. Accordingly, gain and loss figures are taken only on tablets which apparently have a continuous coating.) These were then oven-dried and weighed to measure salt loss. The results are shown in Table III.

TABLE III.—COMPARISON OF DIFFERENT PLASTICIZERS

Plasticizer	% Added to Acetate Phthalate	% Gain After Soaking	% Loss After Drying
Dibutyl tartrate	10	0.9	5
	20	2.4	2.1
	30	2.4	1.2
Diethyl phthalate	10	1.8	2.2
	20	3.5	1.1
	30	1.7	.3
Tripropionin	10	1.4	1.7
	20	1.5	2.7
	30	−1.7	2.4
None	..	−2.2	4.0

These results point to the usefulness of diethyl phthalate in cellulose acetate phthalate coatings to lower the extraction losses of tablets. An additional run was made to compare the coating efficiency of a plasticized and unplasticized solution. One solution contained 45 Gm. of acetate phthalate in 255 Gm. of acetone (15% solids), while the second solution contained 36 Gm. of acetate phthalate and 9 Gm. of diethyl phthalate in the solvent. The latter solution contains 15% nonvolatiles, the plasticizer being at a 20% level based on the finished coating. Each solution was used to coat 500 ten-grain salt tablets, the coating levels being 10, 20, 40, 60, and 100 cc. A 25-tablet sample was taken at each coating level, the sample well dried, and 20 tablets selected for a six-hour soaking in 0.25% HCl. The tablets were weighed and compared in weight to an average value of 13.70 Gm. for 20 uncoated tablets. The soaked tablets were examined to select those without obvious flaws and these were weighed and then well dried and reweighed. Table IV shows the gain and loss data.

At the lower coating levels the solution is apparently not very well distributed. Weighings taken on the "with plasticizer" tablets actually showed values less than the original tablet weight. This might be explained by loss in weight due to grinding off of the salt during tumbling. In these experimental coatings a considerable amount of the coating solids ends up on the sides of the beaker.

It is apparent that with a rather small increase in weight, the tablets are made quite impervious to dilute acid and that diethyl phthalate helps to reduce salt loss.

Tablets coated to the 100-cc. level with plasticized and unplasticized acetate phthalate were tested for solubility in 1.5% sodium bicarbonate (*p*H of 8.6) and in mixtures of 1% Na_2PO_4-H_3PO_4 solutions with *p*H values of 6.8 and 7.5. Tablets in bicarbonate and in the 7.5 *p*H phosphate solution at 98° F. disintegrated in one-half to one hour. The phosphate solution at 6.8 *p*H was slower in action, requiring one to two hours for disintegration. The presence of the plasticizer apparently had no effect on the rate of solution of the coating.

Solubility and *p*H.—The solubilities and compatibilities so far have related to organic solvent systems and have little bearing on the specific properties arising from the free carboxyl group of the combined phthalic acid. In order that the material function as an enteric coating, the acetate phthalate must at least swell in the intestinal fluids so that it can be subsequently broken up. Softening or even complete dissolving of the phthalate does not call for high *p*H solutions but only for solutions which will contribute ions to the phthalate carboxyl and enhance the solubility of the phthalate in water.

Two buffer systems were tried: phosphoric acid-trisodium phosphate and citric acid-potassium citrate. For the phosphate system, 1% solutions of each reagent were made in distilled water. These were then mixed to make 100-cc. quantities of solution, the *p*H was measured and then 0.02 Gm. of powdered acetate phthalate was added and solu-

TABLE IV.—INFLUENCE OF DIETHYL PHTHALATE

	Cc. of Coating Solution	Wt. of 20 Test Tablets	Coating Weight % of Tablet Wt.	"Good" Tablets after 6 Hr.	% of Original Weight	
					6 Hr. in HCl Wiped Dry	Dried at 50° C.
No plasticizer	0	13.70	..	..	..	..
	10	13.80	0.8	0	..	..
	20	13.86	1.2	0	..	..
	40	13.90	1.5	5	..	..
	60	14.12	3.0	17	96	92
	100	14.40	5.0	15	97	94
With plasticizer	10	13.54	..	0	..	..
	20	13.62	..	0	..	..
	40	13.70	..	0	..	..
	60	13.90	1.5	16	100	97
	100	14.22	3.7	20	101	99

bility noted at 98° F. Tests were made on these small samples so that the *p*H of the solution would not change. The solubilities reported in Table V were noted after one and four hours.

Similar work on 1% citrate solutions showed solubility down to a *p*H of 5.9.

TABLE V.—SOLUBILITY IN PHOSPHATE BUFFERS

Cc. 1% Na_2PO_4	Cc. 1% H_3PO_4	*p*H	Solubility 1 Hr.	Solubility 4 Hr.	Final *p*H
45	55	4.9	−	−	..
46	54	5.2	−	−	..
47	53	5.5	−	−	..
48	52	5.8	−	+	5.7
49	51	5.9	−	+	5.8
50	50	6.0	−	+	5.9
60	40	6.7	+	+	6.7
70	30	7.3	+	+	7.3

Water-Soluble Phthalate Salts.—Since cellulose acetate phthalate is effective as an enteric material because it dissolves as a water-soluble salt, it is of interest to study the properties of these salts. The sodium salt of the cellulose acetate phthalate may be prepared by suspending the cellulose ester in distilled water and adding dilute alkali. Weakly alkaline solutions dissolve the phthalate without danger of saponification. When sodium bicarbonate is used, troublesome foaming occurs as the CO_2 is released. This foaming increases as the viscosity of the solution increases. The aqueous sodium salt solution may be used as such, or the salt may be isolated by pouring the solution into excess acetone. The recovered salt is a white powder readily soluble in water.

Typical salt solutions may be prepared as follows: A 100-Gm. sample of cellulose acetate phthalate is slurried in 500 cc. of distilled water. The alkaline agent (9.4 Gm. of sodium hydroxide or 19.8 Gm. of sodium bicarbonate dissolved in 200 cc. of water) is slowly dropped in with stirring. About two hours should be allowed for the addition to prevent ester saponification by the hydroxide. A few drops of capryl alcohol will reduce the foaming in the bicarbonate reaction.

Solutions prepared as above were adjusted to a *p*H of 7 by use of additional base. The acetate phthalate was recovered after three days' standing by pouring the solutions into dilute hydrochloric acid to decompose the salt and washing in changes of distilled water to purify the material. Data in Table VI show these reagents are satisfactory for preparing the sodium salt.

Salts of organic bases may also be prepared. An aqueous solution of the triethanolamine salt may be made by adding the calculated quantity of base in the manner as described above. The salt may also be made in the following manner: 100 Gm. of the cellulose ester is dissolved in 1,800 cc. of acetone.

TABLE VI.—SODIUM SALT PREPARATION

	Reagent Used—NaOH	Reagent Used—$NaHCO_3$
*p*H at end of addition	5.4	5.6
Adjusted *p*H	7.0	7.0
After 3 days	6.3	7.0
Phthalyl analysis on recovered ester	34.8%	34.1%

A solution of 40 Gm. of triethanolamine in 80 cc. of acetone is stirred in. The salt precipitates as it is formed. Vigorous stirring gives a fine powder of the water-soluble salt. The yield is 130 Gm.

Use of the Sodium Salt.—The water-soluble salts may be used as enteric starting materials by mixing their aqueous solutions with medicaments to form a dough-like mass. This composition may be converted to pills or tablets and then dried. Water or dilute alkaline solutions cause rapid disintegration of the tablet, whereas dilute acid converts the salt back to the free acid. In this form the medicament composition is resistant to attack until the acetate phthalate is again converted to a soluble salt in the intestine.

Stability of Salt Solutions.—The aqueous salt solutions of cellulose acetate phthalate are fairly clear and viscous, the viscosity at a given concentration being influenced by the phthalyl content of the ester as well as by the degree of neutralization. Experiments were carried out to determine the stability of solutions prepared at different levels of neutralization. Fifty-gram samples of the cellulose ester were suspended in 250 cc. of distilled water, 8 cc. of *n*-butanol was added to reduce foaming, and the neutralizing agent stirred in. The amount used was calculated from the value of combined phthalyl (35.2%). About one day was required to get good solutions, whereupon the "original" *p*H was measured. The acidity was followed over a period of sixty-four days with samples being recovered for analysis at thirty-two and sixty-four days. These samples were obtained by slowly stirring the salt

TABLE VII.—AGING OF AQUEOUS SALT SOLUTIONS

Degree of Neutralization	*p*H Values of Solutions—Orig.[a]	2 Days	16 Days	32 Days	64 Days	Phthalyl Values 32 Days	64 Days
Pptd. sodium[b] salt	7.2	6.9	5.9	5.5	5.2	30.5	26.9
80% $NaHCO_3$	5.2	5.2	5.0	4.5	4.7	24.8	23.5
100% $NaHCO_3$	7.0	7.2	6.7	6.0	5.6	32.1	28.2
120% $NaHCO_3$	8.0	..	7.0	6.8	6.3	33.0	30.9
TEA[c] salt	5.2	5.2	4.9	4.4	4.6	25.8	22.4
80% TEA	4.9	4.9	4.7	4.4	4.3	25.8	21.1
100% TEA	6.0	5.5	5.3	4.8	4.9	28.9	25.2
120% TEA	7.3	7.2	6.2	5.4	5.3	31.8	29.7
150% TEA	7.6	7.6	6.5	5.6	5.5	31.8	29.7

[a] About twenty-four hours were required to get a good solution.
[b] 1 and 2 neutral salts precipitated, washed, dried, then redissolved in water.
[c] Triethanolamine.

solution into dilute HCl. The precipitate was then thoroughly washed in distilled water. The solutions on standing increased in viscosity and by measurement became more acid and lost phthalyl. It will be noted in Table VII that the overneutralized solutions maintained the highest level of phthalyl. These solutions remained the most fluid throughout the test. Apparently the excess of mild alkali is less of a saponifying agent than the unneutralized carboxyl groups of the combined phthalic acid.

Compatibility of the Sodium Salt.—A stock solution was prepared by dissolving 10 parts of the dried sodium salt in 60 parts of distilled water. Solutions were prepared of other water-soluble polymers and these solutions were added to the stock phthalate salt to give 9:1, 1:1, and 1:9 mixtures. In most cases extra water was needed to make the solutions fluid enough to coat. Compatibility was measured by spreading the solutions in thin layers on a glass plate, allowing the solvent to evaporate, and observing the appearance of the dried film. Films showing no separation of the components were rated as compatible (C). Those showing cloudiness, grain, or phase separation were rated as incompatible (I) (Table VIII).

TABLE VIII.—COMPATIBILITY WITH WATER-SOLUBLE POLYMERS

Test Material	Ratio of Sodium Salt to —Test Material— 9:1	1:1	1:9
"Carbowax" No. 4000	C	I	I
Gelatin	C	C	C
Methocel (medium viscosity)	I	I	I
Polyvinyl alcohol (low viscosity)	I	I	C
Sodium cellulose sulfate	I	I	I
Water-soluble starch	I	I	I

Tolerance for Other Ions.—Aqueous solutions of the phthalate sodium salt when treated with a high concentration of other ions will generally give a precipitate which is the salt of the added ion. Even the sodium salt is "salted out" by high concentrations of soluble sodium salts such as sodium chloride, nitrate, or acetate.

If relatively low concentrations of other ions are added, their solubility effect can best be measured by change in viscosity of the solution. Tests were made wherein 100-cc. portions of a neutral 10% solution of cellulose acetate sodium phthalate were treated with 1, 5, and 10% solutions of various acetate salts. The acetate salts were chosen so that the phthalyl carboxyl would have a chance to capture the added ion. The treated solutions were timed for drainage from a simple pipette of about

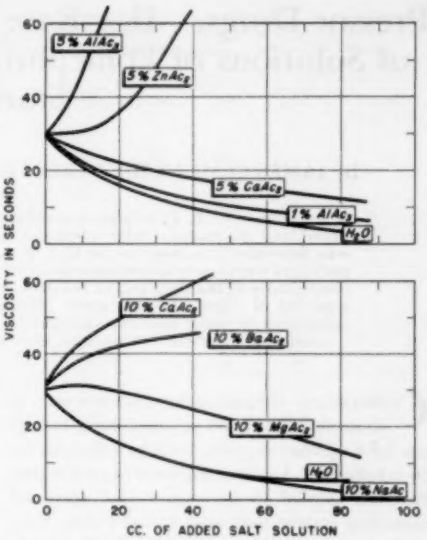

Fig. 3.—Effect of different ions on viscosity of aqueous solutions.

35-cc. capacity and with a 4-mm. opening at the drainage end.

In some cases the added salt, even at 1%, locally precipitates the cellulose ester and does not allow a measurement of change in viscosity. This is true of salts of lead, copper, and mercury. Other salts may be added, and a slow viscosity increase is noted. After a time the salt may precipitate or the solution may gel. Figure 3 shows the viscosity increase caused by certain salts at different concentrations compared with the effect of adding the same amounts of distilled water. These curves indicate the possibility of "bodying" aqueous solutions by careful addition of the proper ions. Aluminum acetate appears to be the best thickening agent. Calcium and barium form a precipitate after a limited viscosity increase.

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Pressor Drugs. II. Rate of Loss in Pressor Potency of Solutions of Epinephrine and Its Analogs During Storage*,†

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A solution of U. S. P. reference standard epinephrine and of an analog were prepared and packaged under identical conditions. The original pressor potency was determined on dogs by the U. S. P. method. Reassays of opened and unopened packages were made following storage of these solutions under refrigeration, at room temperature in the light and in the dark, and under incubation at 37° and at 52° for a period of more than two years. No relation was found between the rate of development of color and any changes in pressor potency. Slight losses developed under any condition of storage, after which a plateau of potency was reached.

COMMERCIAL EPINEPHRINE SOLUTIONS at strengths of 1:1,000 or 1:100 have been in use for a number of years, both by injection and by inhalation. Any manufacturer of such a solution has learned to his sorrow that there are numerous factors that tend to cause loss in activity, as measured by the U. S. P. pressor method against standard *l*-epinephrine made available for this purpose (16, 22). The addition of preservatives such as sodium bisulfite, chlorobutanol, or ascorbic acid, or replacement of air in the filled containers by CO_2, has been widely used as a possible means of protection (1–5, 9–11, 13–15, 18–21, 23, 26).

Our investigations of an analog of epinephrine, "Vaponefrin," indicated that its pressor potency was two-thirds that of *l*-epinephrine base. Other characteristics indicated differences in pharmacodynamic behavior which led to an extensive investigation (17). No changes in potency were observed in the Vaponefrin solutions over periods of five months, whereas U. S. P. standard *l*-epinephrine in the same menstruum and stored under identical conditions showed losses up to one-half of the original activity during a period of two to four weeks. This report contains additional data on the deterioration of Vaponefrin solutions.

A survey of the literature indicates great confusion regarding the quantitative loss in potency of commercial epinephrine solutions. Some authors have found no deterioration over a period of one year or longer, whereas others have reported significant losses in a comparatively short time. Unfortunately data in the literature often failed to indicate the type of menstruum used, the preservative, and the age of the solutions at the time of tests. The U. S. P. (22) requires that epinephrine standard powder be dissolved in 0.1 *N* HCl, diluted to 1:1,000, and preserved in a tightly stoppered hard-glass container, stored under refrigeration and protected from light. This solution must not be used if it shows any change in color, or if it has been prepared more than six months. Under these conditions we have not found any detectable loss in pressor potency of U. S. P. standard epinephrine solutions; in fact many lots have not shown loss in pressor potency in storage under these conditions for periods of two years. The literature reports regarding commercial preparations and their rates of deterioration have been consolidated in Table I (6–8, 12, 24, 25).

EXPERIMENTAL

A single lot of Vaponefrin No. 81839 was prepared and packaged under commercial conditions in one-half oz. amber bottles, sealed with rubber stoppers. Chlorobutanol was present as a preservative and an antioxidant, in a concentration of 0.5%. Determinations of *p*H of different bottles of this lot showed a value of 4.00 by the Beckman glass-electrode method. On October 11, 1939, a group of these unopened bottles was stored under various conditions. At the same time a solution of U.S.P. reference standard epinephrine in the same menstruum, poured into similar bottles under similar conditions, was stored with the Vaponefrin solutions. Storage conditions ranged from an electric refrigerator at 5° to incubation at 55°. At various periods of time, bottles of the reference standard and of Vaponefrin were withdrawn from storage and immediately assayed against freshly prepared U. S. P. reference standard *l*-epinephrine solutions by the U. S. P. pressor method. A smaller number of bottles of the U. S. P. reference standard solution was prepared, and therefore we were not able to extend studies on its deterioration over as long a period as we have for Vaponefrin. The results on the standard are reported in the discussions, but are not included in the tabulations.

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TABLE I.—REPORTED DETERIORATION OF EPINEPHRINE SOLUTIONS 1:1,000

Ref.	Storage Conditions	Time, Mo.	Preservatives Used: None, Full	None, ½ Full	Chlorobutanol	Na_2SO_3	HCl	$Na_2S_2O_5$ Tartrate, Full	$Na_2S_2O_5$ Tartrate, ½ Full
12	Amb. bottles, dark, 10–25°								
	plain	9	68	...	38	50	55	...	...
	paraffin	9	1	...	1.5	2	2	...	...
6, 7	Lab. shelf	4	...	...	...	...	...	4	...
		6	...	...	...	...	3	5	...
		9	...	...	...	...	7	11	...
		12	...	...	...	...	12	21	...
		15	...	...	...	...	18	34	...
		21	...	...	...	...	25	43	...
		27	...	...	...	...	38	56	...
		32	...	...	...	...	45	64	...
		35	...	...	...	...	48	65	...
		42	...	...	...	...	58	69	...
		48	...	...	...	...	65	72	...
24, 25	60-Cc. bottles								
	21°	2	9	16	...	...	...	0	3
	37°	2	20	26	...	...	...	9	15
	2-Cc. ampuls, 21°	2	9	9	...	...	...	5	5
	auto. 21°	2	9	15	...	...	...	5	5
	37°	2	15	20	...	...	...	5	9
8	Tartrate	60	...	...	...	...	...	20	...

A. A series of samples of Vaponefrin and of standard epinephrine solutions was stored in sealed bottles in an electric refrigerator at a temperature of approximately 5°. From time to time a bottle was removed for bioassay; in some instances these opened bottles were discarded, and in other cases they were restoppered, labeled with the date of opening, and returned for further storage. Changes in potencies were determined, giving the data recorded in Table II. Those instances in which previously opened bottles were reassayed are indicated in the table.

There was no significant change in color in the material stored in the refrigerator. There was no loss in pressor potency of the U. S. P. reference standard solution over a period of five months; later assays were not made.

It may be noted that there was no change in potency over a period of three months. There was an apparent increase in potency of 8% in the sample assayed in the eighth month, which was confirmed by a reassay on the same bottle a month later. This was not a common occurrence, although different lots of material packaged in bottles of the same group may often show differences in absolute potency; this may be due to some difference in treatment of the bottle or to some other peculiar factor. Furthermore, tolerances in themselves may account for some of these differences, the U. S. P. having recognized a variation of 5% in either direction as permissible. An additional assay at the end of fifteen months' storage agreed with the results at the end of eight and nine months, as indicating that there was no change in potency in this bottle during the period between the eighth and fifteenth month. This is to be noted in connection with an assay on a different bottle in the thirteenth month, which showed a loss of 8%. This was not

TABLE II.—DETERIORATION OF VAPONEFRIN SOLUTION NO. 81839

Age of Solution, Mo.	Pressor Potency, Conditions of Storage, 10/12/39: A Refrig. Dark, 5°	B Unopened Room Temp., Dark	C Room Temp., Sunlight	D Opened Frequently, Room Temp., Sunlight	E Incub., Dark, 37°	F Incub., Dark, 55°
0	0	0	0	0	0	0
¼	0[a]	0[a]	0[a]	0[a]	0	0
1	0	...	0	0[a]	0	...
2	0[a]	...	...	0[a]	...	...
3	0[a]	0[a]	0	0[a]	0	...
8	+8[b]	...	16[a]	16[a]	+8[b]	...
9	+8[a, b]	...	...	8[a]	+8[a, b]	...
13	8	...	4[a]	16[a]	0	...
15	+8[a, b]	...	+3[b]	16[a]	0[a]	+4[b]
25	...	+4[b]	...	16[a]	...	...
29	...	...	...	16[a]	...	...
40	6[a]	7[a]	12[a]	24[a]	12	22
104	...	...	...	...	16	...
					25[a]	

[a] Bottle reopened.
[b] Gain in potency recorded.

significantly altered by further storage and reassay at the end of a forty-month period. It would appear that changes in color and losses in pressor potency of Vaponefrin would be negligible, following storage in a refrigerator in the dark for periods up to forty months.

B. A group of bottles were placed in a cardboard carton, and stored in a laboratory locker in the dark. The room temperature varied from 20 to 33°. Under these conditions the standard epinephrine solution began to develop some color change in two to four weeks without showing a significant change in the pressor potency. The Vaponefrin solution showed no change in potency over three months; after twenty-five months there was a slight trend for increased potency in a previously unopened bottle, which fell within the usual tolerance for bioassays and was not believed to be significant. The opened bottle reassayed after forty months showed a slight color and precipitate but the loss in potency was only 7%, or substantially the same as that shown by the companion bottle in the refrigerator. Other commercial lots of Vaponefrin have been assayed and reassayed under these conditions of storage at room temperature, with the results set forth in Table III.

TABLE III.—PER CENT LOSS IN PRESSOR POTENCY OF VAPONEFRIN SOLUTIONS (OPENED BOTTLES)

Sample No.	Interval Between Assay Mo.	Loss in Potency, %
6H	24	8
5F	26	0
5C	27	4
4B	27	2
4A1	34	27
4A9	34	20
Unopened Controls		
39	3	0
39	25	+4
4A1	34	9
4A9	34	0
4A9	40	7

C. Bottles of both types of solutions were stored on a laboratory shelf, receiving direct sunlight several hours daily, at room temperature. The standard epinephrine solution showed no significant loss in potency over a period of a month, but did develop very marked color changes and some precipitation leading to the discard of these standard solutions of epinephrine. The Vaponefrin solution showed no significant changes in color over the first year, then developed a light pinkish-brown color and very slight sediment which was somewhat more marked after forty months. As far as the potency is concerned there was no change over the first three months. A previously opened bottle showed a loss of 16% in the eighth month, but another previously opened bottle showed only 4% loss in thirteen months. Still another unopened bottle in fifteen months showed a nonsignificant increase in pressor potency. Reassay of the material after forty months showed a loss of 12% pressor potency which is somewhat greater than that change observed after approximately one year.

From these findings it would seem that storage of epinephrine or Vaponefrin solution in amber bottles will not protect them from eventual losses in potency, or from discoloration. The loss in the potency of the Vaponefrin stored in the sunlight was about twice as much as the loss in solutions stored in the dark at essentially the same temperature.

D. In this portion of the investigation several bottles were placed on a laboratory desk receiving direct sunlight daily. The stopper was removed from each bottle for a period of one minute daily, five days per week for three months, then once weekly for a total period of a year, after which they were opened at less frequent intervals. The standard epinephrine solution showed marked losses in potency and definite discoloration within a period of a week or less; so they were discarded. Somewhat to our surprise there was no loss in potency and no significant change in color in the Vaponefrin solution over a period of three months. By the eighth and ninth month different bottles showed different degrees of discoloration and of loss in potency. The decrease in potency was about 16% in repeated assays over periods of twenty-nine months' storage, and showed 24% after forty months' storage. The latter solution was very dark but had lost relatively less potency than we had expected.

E. A group of bottles was placed in an electric incubator maintained at a temperature of 37°. Samples were withdrawn from time to time for bioassay. The epinephrine showed no changes in color or potency in one week, but developed discoloration within two to four weeks and marked losses in potency which led to its being discarded. The Vaponefrin showed no change in potency over a period of three months; a bottle which showed a somewhat higher value at the eighth month showed no change during an additional month of storage. One bottle of this lot was frequently reassayed; it showed no loss in potency after one week, one month, two months, or thirteen months, nor was there any change in appearance. When opened for the fifteen-month assay there was some darkening in color and some precipitate was noted. When opened for reassay after one hundred and four months' continuous storage at 37°, there was a heavy sludge adhering to the walls of the bottle and the solution was dark in color. The potency assays showed a loss of only 25% for this bottle. Another bottle of the same lot which had never been opened showed somewhat less discoloration and very little precipitation. When a portion of its contents were removed by means of a sterile hypodermic needle, a loss in pressor potency was observed of only 16%, after one hundred and four months of continuous incubation at 37°. This experiment has changed our opinions regarding relationship between storage temperature and loss of pressor potency of Vaponefrin or epinephrine solutions; therefore these two bottles are being held in continuous incubation at 37° for subsequent reassays after five to ten years of additional storage.

F. A further group of bottles of this same lot was stored in an incubator in the dark at 55°, and samples withdrawn from time to time for bioassay. There was no loss in potency and no change in color in the standard epinephrine over a period of one week, but marked changes in color and losses of more than half of the potency developed in one

month, leading to the discarding of the epinephrine material. The Vaponefrin solutions showed no changes in color over a storage period of fifteen months, but marked color and sediment developed during further storage to forty months, in previously unopened bottles. The total loss of potency was 22% in forty months, which was about the same as that noted in the bottles opened frequently and stored in the sunlight at room temperature (*D*). This condition was chosen as representing an extreme of storage, and suggests that no significant changes may be expected in Vaponefrin solution during a period of a year.

The changes in potency during storage of a single lot of Vaponefrin are shown in Table II. Light appears to be a greater factor than temperature in connection with loss in pressor potency.

A series of Vaponefrin solutions were submitted from time to time in the same type of amber bottles, with rubber stoppers or with Bakelite screw tops. These samples have been reassayed after storage at room temperature in the dark for varying lengths of time between twenty-four months and thirty-four months to determine the rate of change in their potency. The reassays on bottles which had been opened are reported in the upper part of Table III, and the assays on the unopened duplicate bottles are reported in the second portion of Table III. It may be noted that the losses in pressor potency over a period of twenty-four to twenty-seven months were negligible, but that significant losses developed thirty-four months after the original opening. With the unopened controls the differences in potency were negligible over an interval up to forty months.

CONCLUSIONS

1. Under identical conditions of storage Vaponefrin was more stable than U. S. P. reference standard epinephrine solution.

2. There was no relationship between changes in color and changes in potency of solutions of standard epinephrine.

3. Solutions of Vaponefrin proved more stable than solutions of U. S. P. standard epinephrine stored similarly and simultaneously.

4. Losses in potency of Vaponefrin solutions in the refrigerator or at room temperature in the dark were insignificant over periods up to three years.

5. When stored in sunlight, losses in potency developed more rapidly; opening bottles further increased the rate of deterioration.

6. Storage in incubators at elevated temperatures showed greater rates of loss than at lower temperatures.

7. Continuous incubation at 37° of Vaponefrin solution for a period of nine years caused losses of not more than one-fourth of the original potency.

8. As far as pressor potency is concerned there is no evidence of serious loss in activity in storage up to nine years; these studies are being extended.

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United States Pharmacopœial Convention

First Annual Financial Statement, Covering the Period from May 1, 1950, to December 31, 1950, and Based upon the Report of the Auditor*

STATEMENT OF INCOME AND EXPENSE (GENERAL FUND)

INCOME				
Sale of Pharmacopœias:				
Collections			$ 212,908.27	
Add: Increasing in Accounts Receivable:				
December 31, 1950	$55,689.69			
April 30, 1950	708.96		54,980.73	$ 267,889.00
Sale of Reference Standards, A. M. A. Articles, etc.				
Collections			$ 10,748.79	
Less: Decrease in Accounts Receivable:				
April 30, 1950	$ 1,668.00			
December 31, 1950	1,379.01			
	$ 288.99			
Wrote Off Cancelled Invoices Applicable to 1948	13.00		275.99	
			$ 10,472.80	
Add: Increase in Collections Pending Remittance to Treasurer:				
December 31, 1950	$ 967.98			
April 30, 1950	859.25			
	$ 108.73			
Less: Adjustment of Prior Year's Collections	.32		108.41	10,581.21
Interest on Investments:				
Collections			$ 5,912.50	
Less: Decrease in Accrued Interest Receivable:				
April 30, 1950	$ 2,087.51			
December 31, 1950	537.47			
	$ 1,550.04			
Add: Amount Accrued to Remington Research Fund at Year Ended April 30, 1950	62.50		1,612.54	
			$ 4,299.96	
Less: Amortization of Bond Premium			37.18	4,262.78
Profit on Sale of Fixed Assets—Net				1,095.64
Use of Text by Others				1,545.00
Miscellaneous				459.15
TOTAL INCOME				$285,832.78
LESS—EXPENSE:				
Revision			$ 66,854.70	
Less: Increase in Inventory:				
December 31, 1950	$18,944.91			
April 30, 1950	11,619.42		7,325.49	$ 59,529.21
Administration				4,311.31
Publication and Sales Expense			$106,774.31	
Less: Increase in Inventory:				
December 31, 1950	$52,794.70			
April 30, 1950	16,127.55		36,667.15	70,107.16
Convention				3,205.92
Headquarters—New York			$ 6,672.43	
Add: New York Office Depreciation Expense:				
Building	$ 1,571.63			
Furniture and Equipment	1,743.22		3,314.85	9,987.28

* The fiscal year will begin on January 1 hereafter.

TOTAL EXPENSE		147,140.88
EXCESS OF INCOME OVER EXPENSE For the Period from May 1, 1950, to December 31, 1950—Exhibit A		$138,691.90

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

CASH ON DEPOSIT, April 30, 1950		$ 27,034.02
ADD—RECEIPTS:		
Sale of Pharmacopœias	$212,908.27	
Sale of Reference Standards, A. M. A. Articles, etc.	10,748.79	
Interest on Investments	5,912.50	
Use of Text by Others	1,545.00	
Sale of Philadelphia Land and Building	8,461.10	
Repayment from Building Fund	17,630.92	
Transferred from Remington Research Fund	2,487.48	
Miscellaneous	531.65	260,225.71
		$287,259.73

LESS—DISBURSEMENTS:

	Revision	Administration	Publications	Convention	Headquarters	Total	
Printing and Binding	.....	.....	$106,774.31	.....	.....	$106,774.31	
Salaries and Honoraria	$40,845.63	$2,113.06	.....	199.07	1,523.83	44,681.59	
Meetings	9,145.42	779.24	.....	1,596.46	.....	11,521.12	
Supplies	1,180.51	93.37	.....	949.92	487.40	2,711.20	
Postage and Telegrams	1,179.98	105.76	.....	87.47	.....	1,371.21	
Utilities	.....	.....	.....	.....	1,123.45	1,123.45	
Taxes	.....	.....	.....	.....	2,916.33	2,916.33	
Insurance	.....	.....	.....	.....	27.78	27.78	
General	14,503.16	1,219.88	.....	375.00	593.64	16,691.68	
Furnishings and Equipment	.....	.....	.....	.....	2,428.28	2,428.28	
	$66,854.70	$4,311.31	$106,774.31	$3,205.92	$9,100.71		190,246.95
CASH ON DEPOSIT, December 31, 1950							$ 97,012.78

A MORE DETAILED AND SUPPLEMENTARY STATEMENT OF THE REVISION EXPENSES

REVISION SALARIES AND HONORARIA		
Salaries	$23,820.63	
Honoraria	17,025.00*	$40,845.63
REVISION MEETINGS		
Revision Committee	$ 7,307.97	
Committees, Boards, Conferences	1,837.45	$ 9,145.42
REVISION GENERAL		
Reference Standards	$ 6,546.39	
Extending Information Booklets, etc.	1,264.30	
Retirement	5,284.00	
Research	500.00	
Air Travel Card Deposit	425.00	
Miscellaneous	483.47	$14,503.16

* For preparation of U. S. P. XIV—Distributed to the following:

Walter A. Bastedo	M. S. Dooley	Robert W. Heinle	George W. McCoy	Hugo H. Schaefer
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Details of distribution may be had upon request.

Note

The Synthesis of Acetyl-1-C^{14}-Salicylic Acid*,†

By JEAN W. CLAPPISON, J. P. HUMMEL, W. D. PAUL, and J. I. ROUTH

IN STUDIES of salicylate metabolism, the rate of *in vivo* hydrolysis of the acetate portion of acetyl salicylate was of interest. The results of experiments using acetyl-1-C^{14}-salicylic acid, which was prepared by the following procedure, will be reported in a later publication.

The method of synthesis employed was convenient for small-scale preparations of C^{14}-labeled aspirin in good yield without elaborate laboratory facilities. Sodium acetate-1-C^{14} was converted to acetyl chloride which was allowed to react with salicylic acid in the presence of dimethyl aniline.

$$CH_3C^{14}OONa \xrightarrow{C_6H_5OCl} CH_3C^{14}OCl \xrightarrow{C_6H_4OHCOOH} HOOC\text{-}C_6H_4\text{-}OC^{14}OCH_3$$

EXPERIMENTAL

One millimole of sodium acetate-1-C^{14} with an activity of 1 mc./mM[1] dissolved in 20 ml. of water was diluted with 16.6 Gm. of $NaC_2H_3O_2 \cdot 3H_2O$ in a large casserole. The mixture was cautiously heated in an oil bath to remove most of the water and then partially fused by heating over a low flame. The diluted anhydrous sodium acetate was stored in a tightly stoppered bottle.

A distilling flask† (150-cc.) with a 20-cm. fractionating column was directly fitted by all glass connection to a 100-ml. three-neck round-bottom flask with an Allihn condenser and a mercury seal stirrer. The round-bottom flask, containing 9.2 Gm. of dry salicylic acid and 10 cc. of redistilled dimethyl aniline in 50 ml. of absolute ether, was cooled in an ice bath. Five grams of the diluted anhydrous sodium acetate-1-C^{14} was placed in the distilling flask, together with 75 ml. of redistilled benzoyl chloride (a 960 mole per cent excess).

The distilling flask was gradually heated in an oil bath. The acetyl chloride-1-C^{14} began to distill at 49° when the bath temperature had reached 170°. The temperature was slowly raised to 225° over a thirty-minute period during which the bulk of acetyl chloride distilled into the ice-chilled round-bottom flask containing the salicylic acid and dimethyl aniline. The yield of acetyl chloride in this step had been previously estimated to be approximately 85%; the amounts of salicylic acid and dimethyl aniline employed were calculated on that basis. A reaction took place immediately, as evidenced by the separation of a layer of dimethyl aniline hydrochloride. After stirring an additional fifteen minutes after distillation ceased, the ether layer was decanted and the dimethyl aniline hydrochloride layer was shaken with ether three times. The combined ethereal solutions were dried over Na_2SO_4 and then concentrated to approximately 20-ml. volume. An excess of low-boiling petroleum ether was added to precipitate the acetyl salicylic acid and crystallization was complete after standing overnight at −15°.

The product after recrystallization from ether-petroleum ether was 7.6 Gm. (69% yield). When compared with an authentic sample of acetyl salicylic acid by means of infrared spectrophotometry, the radioactive compound exhibited an identical pattern in the range of 2–15 μ. The radioactivity of the product as measured with a 1.4 mg./cm.[2] mica window counter at a distance of 6 mm. was 2,990 impulses/mg./min.

SUMMARY

Acetyl-1-C^{14}-salicylic acid was prepared from sodium acetate-1-C^{14} by formation of acetyl-1-C^{14}-chloride which reacted with salicylic acid in the presence of dimethyl aniline.

* Received June 6, 1951, from the Departments of Biochemistry and Internal Medicine (Div. Physical Med.), State University of Iowa Medical School.

† Aided in part by grants from Bristol-Myers Company, New York, and the Institute for the Study of Sedative and Analgesic Drugs.

[1] Obtained from Tracerlab, Inc.

Book Reviews

The Pharmacologic Principles of Medical Practice. 2nd ed. By JOHN C. KRANTZ, Jr., and C. JELLEFF CARR. The Williams and Wilkins Co., Baltimore, 1951. xvii + 1116 pp. Illustrated. 16 x 23.5 cm. Price $10.

This splendid text is as up-to-date as the latest pertinent literature at the time the manuscript went to press. The same readable style that characterized the first edition is retained, and much new information is available in the authors' clear and concise presentation.

In order to present the pharmacodynamic and pharmacotherapeutic actions of drugs as they are used in medicine, extensive revisions in nearly all of the chapters in the text are evident. This was necessary because of the rapid advances which have been made in pharmacology in the past few years. The chapter on the antibiotics includes aureomycin, chloramphenicol, and terramycin, in addition to other new but less widely used antibiotics. New chapters discuss "The Chemotherapy of Tuberculosis," "The Chemotherapy of Rickettsial Diseases," "Anti-Motion Sickness Drugs," and "Treatment of the Arthritides; the Adrenal Corticotropic Hormone and Cortisone."

The text material has been correlated with the latest official drug compendia: the U. S. P. XIV and the N. F. IX, and with N. N. R. 1950. The review of the first edition of the text clarified an ambiguous reference to the National Formulary. The second edition contains a perfectly clear and correct statement about that official publication.

As was stated in the review of the first edition of the text by Drs. Krantz and Carr [THIS JOURNAL, **38**, 416(1949)]: "Probably no supporting science to the field of medicine has made greater progress during the past decade than pharmacology. Not only have a wealth of new drugs entered the picture, but the drugs have been of such a nature that whole concepts of drug action have been changed or, at least, have been modified. Because of these advances, a new textbook has been badly needed and Drs. Krantz and Carr have met that need very well.

"The authors have consistently emphasized their theme of pharmacologic *principles* in medical *practice*. Never is basic principle sacrificed for obscure detail and yet rarely absent is the detail necessary for intelligent practice. In addition, the facts are marshaled in a readable and teachable manner. The human interest approach is used throughout and given emphasis by an excellent collection of portraits of the leaders in the field—Magendie, Abel, Fleming, Spalding (too few pharmacologists appreciate his contribution), A. J. Clark, Waksman, Domagk, Wells, Sertürner, Chen, Hunt, McCollum, and others.

"The drugs are grouped according to physiologic processes into anti-infective drugs, the response of skin and mucous membranes to drugs, the response of the central nervous system, the autonomic nervous system, the heart and circulation, and the reproductive system to drugs, and the effect of pharmacologic agents on metabolism.

"The appendix carries an interesting section on the discovery and evaluation of new drugs as well as a series of typical prescriptions."

Soybeans and Soybean Products. Vol. II. Edited by KLARE S. MARKLEY. Interscience Publishers, Inc., New York, 1951. xvii + 604 pp. Illustrated. 16 x 23.5 cm. Price $11.

The first volume of this book was reviewed earlier [THIS JOURNAL, **40**, 116(1951)]. Since there are so many ramifications to the soybean industry and such a large number of products involved, the writing of a comprehensive review of the second volume will not be attempted. The contents of this volume, reproduced below, will indicate its scope. The quality of the several additional chapters is assured from the names of the contributors.

C. Processing.

15. Solvent Extraction Processes (Louis F. Langhurst).

D. Utilization of Soybean Products.

16. Production and Utilization of Lecithin (Joseph Stanley).
17. Processing of Edible Soybean Oil (J. W. Bodman, E. M. James, and S. J. Rini).
18. Nutritional Value of Soybeans and Soybean Products (Harry J. Deuel, Jr.).
19. Edible Soybean Oil Products (H. C. Black and K. F. Mattil).
20. Soybean Oil By-Products (R. W. Lehman and N. D. Embree).
21. Nonedible Soybean Oil Products (Theodore F. Bradley).
22. Soybean Oil Meal for Livestock and Poultry (J. W. Hayward).
23. Soybean Protein Food Products (R. S. Burnett).
24. Soybean Protein Industrial Products (R. S. Burnett).
25. Other Soybean Products (Janice M. Smith and Frances O. Van Duyne).

The pagination for volumes I and II of *Soybeans and Soybean Products* is continuous, and excellent author and subject indexes to both volumes are included at the end of the second volume.

An Introduction to Organic Chemistry. 7th ed. By ALEXANDER LOWY, BENJAMIN HARROW, and PERCY M. APFELBAUM. John Wiley & Sons, Inc., New York, 1951. xiv + 480 pp. Illustrated. 14.5 x 22 cm. Price $5.

This readable and interesting text should serve as a valuable introductory guide to organic chemistry. It already has served this purpose for many students since its first edition was printed in 1924.

This seventh edition has required extensive revisions, especially in the sections devoted to aliphatic hydrocarbons and polymers. The correlation of the subject matter with medical and biological applications and the inclusion of pictures of historically important chemists adds to the value of the text.

The use of heavy single lines to indicate double bonds in cyclic compounds, especially with aromatic nuclei, is very intriguing and has possibilities. However, the present application of this principle is an outstanding flaw in an otherwise excellent book. In some formulas the benzene ring would appear to have six double bonds or five or four double bonds instead of three, because all of the lines are of approximately the same thickness and darkness. This would create lasting but false impressions in the mind of a student. The impression would be lasting because this is a most attractive ring presentation, and for this reason the proof should have been studied with extreme care in order to correctly represent the valence bonds.

Much information has been tabulated, and an exceptionally useful table of organic type formulas is included. This latter table should be a splendid memory aid for the student of elementary organic chemistry. Inclusion of the American Chemical Society nomenclature of organic compounds, Latin and Greek roots used in chemical terminology and a comprehensive bibliography are additional features of the book.

Substances Naturelles de Synthese. Vols. I and II. By LEON VELLUZ. Masson et Cie, Paris, 1951. 141 pp. and 138 pp. 16 x 23.3 cm.

These volumes are the collaborative products of Drs. J. Mathieu and L. Velluz and chemical engineers A. Petit and P. Poirier, with Dr. L. Velluz as the editor. The authors present the published procedures used in the syntheses of certain compounds. The most important synthesis for each compound is presented in detail, giving the individual steps for the intermediate preparations. Other methods of syntheses for the same compounds are summarized. Information obtained during actual synthesis is included in the notes that are given for each compound. Alternate procedures are given where they have been found to be more efficient or more feasible.

The volumes are similarly divided into three sections. Section I is devoted to "Preparations" with a chapter given to each compound. The compounds included are: (Vol. I) Ascorbic Acid, Adenine, marked Adenine (N^{15} at positions 1 and 3), Adenosine, Chloramphenicol, Esculoside, DL-Histidine, L-Tryptophane; (Vol. II) Adenylic Acid, DL-Aspartic Acid, Desoxycorticosterone, DL-Lysine, DL-Methionine, Progesterone, L-Threonine, L-Thyroxin. Section II is devoted to special methods of synthesis: (Vol. I) Coumarinic and Pyrimidic Cyclization; (Vol. II) Oppenauer Reaction and Functional Separation of Carbonyl Derivatives. The cyclization reactions are tabulated to show the reacting materials, the products, and the literature references. Section III is devoted to practical notes: (Vol. I) Pure Solvents (preparation, tests, and dehydrating agents); (Vol. II) Carbonyl Reagents, Ketone Reactions.

The text is written in a readable French style, but an English translation would enlarge its field of usefulness. Each volume is well indexed according to the compounds and their intermediates, in addition to alphabetical lists of compounds and authors of references.

This series, which will be continued, is a splendid addition to the literature on modern organic synthesis.

BOOKS RECEIVED

Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales. Vol. VIII, No. 30, April, 1951. Publicación del Ministerio de Educación Nacional, Bogotá, Colombia. 143 pp. Illustrated. 25 x 33 cm.

The Enzymes—Chemistry and Mechanism of Action. Vol. II, Part 1. Edited by JAMES B. SUMNER and KARL MYRBÄCK. Academic Press, Inc., New York, 1951. xi + 790 pp. 16 x 24 cm. Price $14.80.

Biochemistry and Physiology of Protozoa. Vol. I. By ANDRÉ LWOFF. Academic Press, Inc., New York, 1951. x + 434 pp. 15.5 x 23.8 cm. Price $8.80.

Vitamin Methods. Vol. II. Edited by PAUL GYÖRGY. Academic Press, Inc., New York, 1951. xi + 740 pp. Illustrated. 16 x 23.5 cm.

Metabolic Methods (Clinical Procedures in the Study of Metabolic Functions). By C. FRANK CONSOLAZIO, ROBERT E. JOHNSON and EVELYN MAREK. C. V. Mosby Company, St. Louis, 1951. 471 pp. Illustrated. 18 x 25.5 cm. Price $6.75.

Radioactivity Applied to Chemistry. Edited by ARTHUR C. WAHL and NORMAN A. BONNER. John Wiley & Sons, Inc., New York, 1951. xv + 604 pp. 15.5 x 23.5 cm. Price $7.50.

Physical Biochemistry. 2nd ed. By HENRY B. BULL. John Wiley & Sons, Inc., New York, 1951. ix + 355 pp. 15.5 x 23.7 cm. Price $5.75.

Solutions and Dosage. 2nd ed. By SARA JAMISON. McGraw-Hill Book Co., Inc., New York, 1951. xiii + 163 pp. 22 x 28 cm. Price $2.25.

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